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Programa de Doctorado: **Biociencias y Ciencias Agroalimentarias**

RESISTENCIA A GLIFOSATO EN EL GÉNERO *CHLORIS* Y *PARTHENIUM* EN LATINOAMERICA. MECANISMOS DE RESISTENCIA Y CONTROL ALTERNATIVO

RESISTANCE TO GLYPHOSATE IN CHLORIS AND PARTHENIUM SPP. IN LATIN AMERICA. MECHANISMS OF RESISTANCE AND ALTERNATIVE CONTROL

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TITULO: *RESISTENCIA A GLIFOSATO EN EL GÉNERO CHLORIS Y PARTHENIUM EN LATINOAMERICA. MECANISMOS DE RESISTENCIA Y CONTROL ALTERNATIVO*

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TÍTULO DE LA TESIS

RESISTENCIA A GLIFOSATO EN EL GÉNERO *CHLORIS* Y *PARTHENIUM* EN LATINOAMERICA. MECANISMOS DE RESISTENCIA Y CONTROL ALTERNATIVO

DOCTORANDO:

ENZO RICARDO BRACAMONTE

LOS DIRECTORES DE LA TESIS INFORMAN:

Que el presente trabajo de investigación titulado

"Resistencia a glifosato en el género *Chloris* y *Parthenium* en latinoamerica. Mecanismos de resistencia y control alternativo"

constituye la memoria que presenta D. Enzo Ricardo Bracamonte para aspirar al grado de **Doctor en Biociencias y Ciencias Agroalimentarias** siendo realizado en el laboratorio del Departamento de Química Agrícola y Edafología de la Universidad de Córdoba, y en el laboratorio de Genómica Funcional del Departamento de Mejora Genética Vegetal del Instituto de Agricultura Sostenible (IAS-CSIC, Córdoba), bajo nuestra dirección y supervisión. Consideramos que el Doctorando cumple los requisitos legales para optar al grado de **Doctor en Biociencias y Ciencias Agroalimentarias**.

A continuación, se presenta una relación de los trabajos publicados a los que ha dado lugar la investigación realizada y que forma parte del cuerpo de la Tesis:

Bracamonte, E., Silveira, H.M.D., Alcántara-de la Cruz, R., Domínguez-Valenzuela, J.A., Cruz-Hipolito, H.E., De Prado, R. From tolerance to resistance: mechanisms governing the differential response to glyphosate in *Chloris barbata* (2018) ***Pest Management Science***, 74 (5), pp. 1118-1124. DOI: 10.1002/ps.4874 ISSN: 1526498X IF 3.253

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Por todo ello, se autoriza la presentación de la Tesis Doctoral.

Córdoba, Septiembre de 2018.

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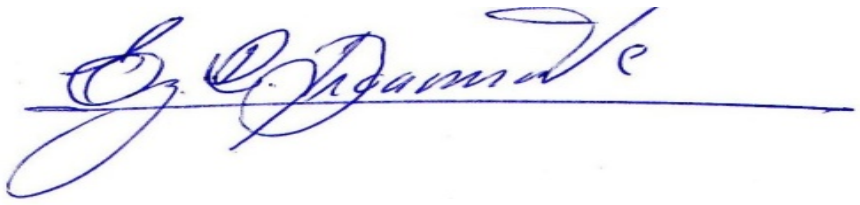
RESISTENCIA A GLIFOSATO EN EL GÉNERO *CHLORIS* Y *PARTHENIUM* EN LATINOAMERICA. MECANISMOS DE RESISTENCIA Y CONTROL ALTERNATIVO

Trabajo presentado para optar al grado de
Doctor en Biociencias y Ciencias Agroalimentarias



Fdo.: **Enzo R. Bracamonte**
Ing. Agrónomo MSc

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Mi tesis la dedico a los amores de mi vida, mis queridos hijos Sofía y Matias, por darme la oportunidad de conocer la belleza de la vida.

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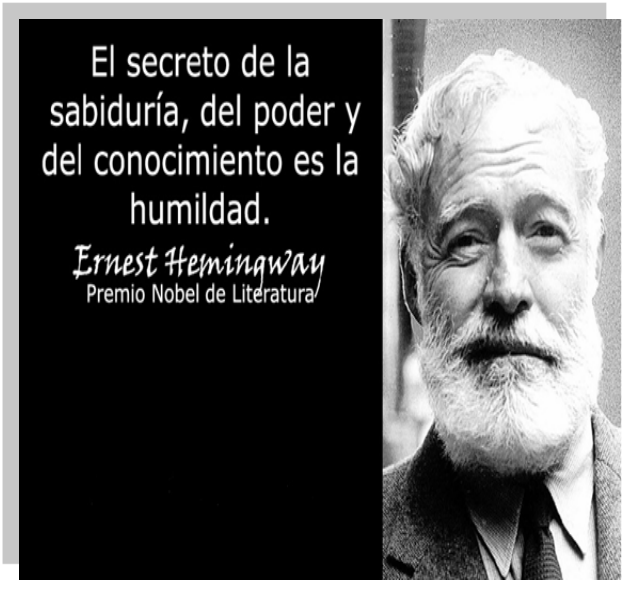
Con todo mi corazón

Enzo



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Resistencia a Glifosato en Poblaciones de *Chloris spp.* en Argentina y alternativas de Control Químico

Lista de Abreviaturas

^{14}C	Carbono14
AAPRESID-REMA	Asociacion Arg. De productores en siembra directa-Registro Nacional de malezas resistentes
ACCasa	Acetil coenzima A carboxilasa
ACCase	Acetyl CoA Carboxylase
ae	Ácido equivalente
ALS	Acetolactato sintasa
AFLP	Polimorfismos en la longitud de fragmentos amplificados
AMOVA	Análisis de varianza molecular
ANOVA	Análisis de la varianza
AMPA	Ácido aminometilfosfónico
bp	Par (es) de base (s)
C4	Via fotosintética de 4 carbonos
cDNA	ADN complementario de la primera cadena
DAT/DDT	Días después del tratamiento
CIM/MIM	Control Integrado de Malas hierbas/malezas
DAT/DDA	Days after treatment
DF	Degrees of freedom
DNA	Acidodesoxirribonucleico
ED/GR_{50}	Dose causing 50% reduction in growth
EDTA	Acido etilendiaminotetraacético,
EPSPS	5-enolpiruvatoshiquímato-3-fosfato sintasa
g	Gramo (s)
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
FR	Resistant Factor
g ae ha^{-1}	Grams of acid equivalent per hectare
g ai ha^{-1}	Grams of active ingredient per hectare
GOX	Glyphosate oxidoreductase
Ha	Hectárea
HAT	Hours after treatment.
HDT	Hora (s) después del tratamiento
HRAC	Herbicide Resistance Action Commitee
I_{50}	Dose causing 50% inhibition of herbicide activity
IWM	Integrated Weed Management
kBq	Kilobequerelio
LSS	Espectrometría de centelleo líquido
kBq	Kilobeq
LD_{50}	The herbicide dose causing 50% mortality
MBq	Megabequerelio
mM	milimol

MOPS	Buffer : 3-(*N*-morpholino)propanesulfonic acid)
n	Tamaño de muestra
ng	Nanogramo (s)
nd	No detectado
Ne	Número de alelos efectivos
nm	Nanómetro (s)
NTSR	Non-Target Site Resistance
NTSR	Resistencia fuera del sitio de acción
PCR	Reacción en cadena de la polimerasa
pH	Potencial de hidrógeno
qPCR	PCR cuantitativa
PPO	Protoporphyrinogen Oxidase
R	Poblacion resistente a herbicida
RI	Índice de resistencia
RNA	Ácido ribonucleico
RT	Índice de tolerancia
TSR	Resistencia en el sitio de acción herbicida
S	Poblacion no resistente o sensible a herbicida
SENASA	Servicio Nacional de Sanidad y Calidad Agroalimentaria de Argentina
Soja GR	Soja resistente a glifosato
Soja RR	Soja Roundup Ready
TSP	Proteina trombospondina
TSR	Target Site Resistance
UPGMA	Grupo de pares no ponderados con medias aritméticas
USDA	Departamento de agricultura de EEUU
v/v	Volumen en relación a volumen
w/v	Peso en relación a volumen
WSSA	Weed Science Society of America
µg	Microgramo
µL.	Microlitro
µM	Micromol

RESUMEN

La incidencia nociva de las malas hierbas constituye uno de los mayores obstáculos para alcanzar producciones agrícolas sustentables, produciendo pérdidas de hasta el 10% y más del 30% de las cosechas en los países desarrollados y en desarrollo, respectivamente. El control de malezas mediante el uso predominante de herbicidas produjo la aparición y difusión exponencial de biotipos tolerantes y resistentes a las principales familias químicas utilizadas. En Latinoamérica desde hace casi 40 años, las poblaciones de malas hierbas están bajo presión de selección continua por el uso de glifosato. Entre las especies que están incrementado en forma significativa su presencia en esta región podemos citar a *Parthenium hysterophorus* L y el género *Chloris*. *Parthenium hysterophorus* L. (Asteraceae, Heliantheae), es una planta herbácea anual o de corta duración nativa de la región del Golfo de México. Es considerada una de las peores malezas debido a su capacidad de invasión, potencial de propagación, impacto económico y sobre la salud humana y el ambiente. Debido a su alto potencial de invadir y perpetuarse sobre suelo no disturbados torna a *Parthenium hysterophorus* como una seria amenaza en sistemas agrícolas con grandes superficies bajo siembra directa como Brasil y Argentina. Dentro del género *Chloris* existen al menos 60 especies en Latinoamerica, incluyendo al género *Trichloris* y otras especies de la tribu de las Chlorideas. Estas especies aumentaron considerablemente su difusión en cultivos perennes, entre ellos los frutales cítricos con amplio uso del herbicida glifosato y en países con amplia adopción del sistema tecnológico siembra directa-soja RR (Roundup Ready)-glifosato, como Brasil y Argentina. Estos sistemas productivos propiciaron el aumento de la presión de selección y por ello, la difusión de biotipos resistentes a este principio activo como *Chloris barbata*, *Chloris elata* y *Chloris virgata.* Estas especies no son homogéneas en cuanto su distribución geográfica, taxonomia ni en su respuesta a herbicidas. Esta situación complica significativamente su estudio y su manejo con alternativas de control eficientes y homogéneas. Por ello, la caracterización morfológica mediante estudios de diferenciación de especies y la determinación de los mecanismos de resistencia al herbicida glifosato constituyen herramientas imprescindibles para poder establecer estrategias de manejo eficientes, integradas y sustentables de *Parthenium hysterophorus* L y de especies de *Chloris* presentes en áreas agrícolas de Latinoamérica. En relación a lo anteriormente descripto es que se plantea la justificación del este trabajo de investigación y los objetivos propuestos.

ABSTRACT

The harmful incidence of weeds is one of the biggest obstacles to achieving sustainable agricultural production, producing losses of up to 10% and more than 30% of the harvests in developed and developing countries, respectively. The control of weeds by the predominant use of herbicides produced the appearance and exponential diffusion of tolerant and resistant biotypes to the main chemical families used. In Latin America for almost 40 years, weed populations are under constant selection pressure due to the use of glyphosate. Among the species that are significantly increased their presence in this region we can mention *Parthenium hysterophorus* L and the genus *Chloris*. *Parthenium hysterophorus* L. (Asteraceae, Heliantheae), is an annual or short-lived herbaceous plant native to the Gulf of Mexico region. It is considered one of the worst weeds due to its ability to invade, potential for propagation, economic impact and on human health and the environment. Due to its high potential to invade and perpetuate on undisturbed soil makes *Parthenium hysterophorus* a serious threat in agricultural systems with large areas under direct seeding such as Brazil and Argentina. Within the genus *Chloris* there are at least 60 species in Latin America, including the genus *Trichloris* and other species of the Chlorideas tribe. These species increased considerably their diffusion in perennial crops, among them the citrus fruit trees with ample use of the herbicide glyphosate and in countries with wide adoption of the direct-soybean RR (Roundup Ready) -glyphosate technology package, such as Brazil and Argentina. These productive systems favored the increase of selection pressure and, therefore, the diffusion of biotypes resistant to this active principle such as *Chloris barbata*, *Chloris elata* and *Chloris virgata*. These species are not homogenous in terms of their geographical distribution, taxonomy or in their response to herbicides. This situation significantly complicates its study and its management with efficient and homogeneous control alternatives. Therefore, the morphological characterization through studies of species differentiation and the determination of glyphosate herbicide resistance mechanisms are essential tools to establish efficient, integrated and sustainable management strategies of *Parthenium hysterophorus* L and *Chloris* species present in agricultural areas of Latin America. In relation to the previously described is that the rationale of this research work and the objectives proposed.

OBJETIVOS

Los Objetivos propuestos en esta tesis son:

- Identificar los mecanismos de resistencia NTSR y TSR involucrados sobre el nivel de resistencia de *Parthenium hysterophorus* a glifosato. Recogido en: **Bracamonte, E.**, Fernández-Moreno, P.T., Barro, F., de Prado, R. Glyphosate-resistant *Parthenium hysterophorus* in the Caribbean Islands: Non target site resistance and target site resistance in relation to resistance levels (2016) ***Frontiers in Plant Science***, 7, art. no. 1845. DOI: 10.3389/fpls.2016.01845 ISSN: 1664462X.

- Determinar la identidad taxonómica de tres especies diferentes de *Chloris* sospechosas de resistencia en Cuba mediante análisis morfológicos y moleculares. Recogido en: **Bracamonte, E.R.**, Fernández-Moreno, P.T., Bastida, F., Osuna, M.D., Alcántara-De La Cruz, R., Cruz-Hipolito, H.E., De Prado, R. Identifying *Chloris* species from cuban citrus orchards and determining their glyphosate-resistance status (2017) ***Frontiers in Plant Science***, 8, art. no. 1977. DOI: 10.3389/fpls.2017.01977 ISSN: 1664462X.

- Determinar y evaluar los niveles de resistencia/tolerancia y los mecanismos involucrados de tres especies de *Chloris* sospechosas de resistencia en Cuba. Recogido en: **Bracamonte, E.R.**, Fernández-Moreno, P.T., Bastida, F., Osuna, M.D., Alcántara-De La Cruz, R., Cruz-Hipolito, H.E., De Prado, R. Identifying *Chloris* species from cuban citrus orchards and determining their glyphosate-resistance status (2017) ***Frontiers in Plant Science***, 8, art. no. 1977. DOI: 10.3389/fpls.2017.01977 ISSN: 1664462X.

- Determinar y evaluar los niveles de resistencia/tolerancia y los mecanismos de resistencia involucrados de dos poblaciones de *Chloris barbata* sospechosas de resistencia a glifosato en Mexico. Recogido en: **Bracamonte, E.**, Silveira, H.M.D., Alcántara-de la Cruz, R., Domínguez-Valenzuela, J.A., Cruz-Hipolito, H.E., De Prado, R. From tolerance to resistance: mechanisms governing the

differential response to glyphosate in *Chloris barbata* (2018) ***Pest Management Science***, 74 (5), pp. 1118-1124. DOI: 10.1002/ps.4874 ISSN: 1526498X

- Evaluar la respuesta de control y nivel de resistencia de *Chloris virgata y Chloris elata* presentes en campos agrícolas de Argentina expuestos a glifosato mediante ensayos de prospección dosis-respuestas en invernadero y campo.

- Establecer estrategias de manejo alternativas, integradas y sustentables de las especies de *Chloris* presentes en áreas agrícolas de Latinoamérica.

CAPÍTULO I

INTRODUCCIÓN GENERAL

Las Malas Hierbas

La agricultura es una actividad de gran importancia para el desarrollo y riqueza de las naciones, y su práctica se ve afectada por diversos factores bióticos y abióticos que causan grandes pérdidas en el rendimiento de los cultivos (Oerke, 2006). Considerando que las interacciones entre cultivos y las malas hierbas reducen la producción primaria neta de los cultivos, los agroecosistemas están constantemente sometidos a flujos de energía en forma de subsidios que intentan minimizar esas interacciones biológicas negativas (Vila-Aiub, 2008).

Las malas hierbas ocasionan pérdidas directas cuando compiten con los cultivos cuando los recursos luz, agua y nutrientes son escasos. La incidencia nociva de las malas hierbas constituye uno de los mayores obstáculos para alcanzar producciones agrícolas sustentables en el mundo, produciendo pérdidas de hasta el 10% y hasta 60 % de las cosechas en los países desarrollados y en desarrollo, respectivamente (Pimentel *et al*. 2001). Además, las malas hierbas causan daños indirectos ya que dificultan las labores de recolección de los cultivos y pueden ser reservorio de plagas y enfermedades que afectan a los cultivos (Liebman *et al.* 2001).

De las 250.000 especies vegetales existentes, aproximadamente 8.000 (3%) son consideradas malas hierbas y 250 sps. son problemáticas, representando el 0.1% de la flora mundial. El 70% de las malezas-problema corresponden a 12 familias botánicas y el 40% pertenecen a 2 familias: *Poaceae* y *Asteraceae*, presentándose la misma concentración de familias que en la situación de los cultivos más importantes.

La Malherbología es la ciencia que estudia la biología, ecología y manejo de malezas. De acuerdo a la mayor parte de la literatura, se puede definir a la mala hierba o maleza* como "una planta que crece en un momento y en un lugar no deseado".

Desde un punto de vista antropocéntrico se considera maleza al conjunto de ellas, que crece donde no es deseada y que afecta los intereses del hombre. Pujadas y Hernández (1998) definieron "mala hierba" como; *Aquella planta que crece siempre, o de forma predominante, en situaciones marcadamente alteradas por el hombre y que resulta no deseable por él en un lugar y un momento determinados.* Según la definición anterior, cualquier planta puede comportarse como "mala hierba".

.....

En el presente trabajo se utilizará de manera indistinta los términos mala hierba y maleza

Taxonómicamente las malezas no pertenecen a ningún taxón botánico específico. Es importante destacar que de 12 cultivos pertenecientes a cinco familias botánicas aportan el 75% del alimento mundial, y a esas mismas cinco familias pertenecen muchas de las peores malezas. Esto implica que nuestros principales cultivos y malezas comparten características taxonómicas y probablemente un origen común (Cobb, 1992).

Los métodos de control de malezas se clasifican dependiendo del autor y del momento histórico considerado. Actualmente, el método de control químico basado en el uso de herbicidas constituye el más utilizadas en la agricultura intensiva moderna.

Los herbicidas pueden ser clasificados de acuerdo a varios criterios. Actualmente, el uso de herbicidas sigue criterios técnicos y científicos y se basa en la elección de los principios activos considerando la familia química y los mecanismos y modos de acción involucrados en el control.

La clasificación basada en la estructura química de los diferentes compuestos usados como herbicidas considera que sus integrantes poseen propiedades químicas similares, y generalmente tienen el mismo modo de acción (Retzinger y Mallory-Smith, 1997; Hance y Holly, 1990).

La clasificación más útil de los herbicidas es por su modo de acción (Duke y Dayan, 2011; HRAC, 2016). El modo de acción es la secuencia de eventos que ocurren desde la absorción del herbicida hasta la muerte de la planta (Figura 1).

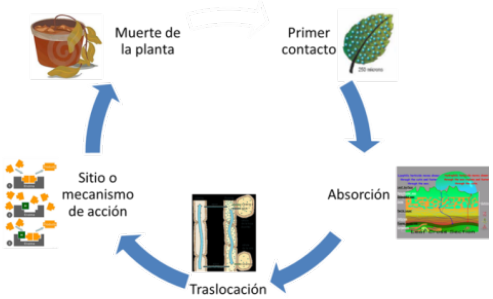


Figura 1. Representación esquemática del modo de acción de un herbicida.

Los herbicidas con el mismo modo de acción tienen el mismo comportamiento de absorción y transporte, y producen síntomas similares en las plantas tratadas (Gunsolus y Curran, 1996). Esta clasificación de los herbicidas permite predecir, en forma general, su espectro de control de maleza, fitotoxicidad, época de aplicación, selectividad a cultivos y persistencia en el suelo. Ashton y Craft (1981); Cobb y Reade (2010), distinguen siete grandes grupos, dentro de los cuales a su vez se incluyen uno o más mecanismos de acción. Actualmente, de los 26 modos de acción conocidos, solo 6 de ellos explican el 80% del mercado de herbicidas (17 billones de U$S anuales).

El mecanismo de acción de un herbicida, se define como la principal reacción bioquímica o biofísica que es afectada por el herbicida en la planta tratada. El mecanismo de acción comúnmente incluye el efecto de algún proceso enzimático vital para la planta. Los herbicidas pueden funcionar de diferentes maneras en la planta, interfiriendo con algún proceso esencial para su correcto crecimiento y desarrollo de la planta (Figura 2).

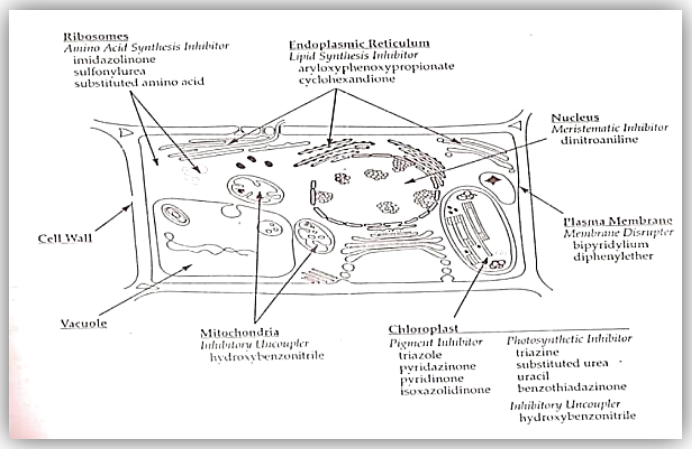


Figura 2. Sitios primarios involucrados en los principales mecanismos de acción de los herbicidas.

Los herbicidas pueden ser clasificados por su mecanismo de acción, con base en los síntomas provocados por éstos en las plantas tratadas. La Sociedad Americana de la Ciencia de la Maleza (WSSA, 1998) ha desarrollado un sistema de clasificación basado en numeración arábiga, mientras que la clasificación del Comité de Acción de la Resistencia a Herbicidas (HRAC, 2016) se basa en letras. El uso de estos parámetros ayuda a comprender y mejorar la eficiencia de control, considerando aspectos

fisiológicos y metabólicos (selectividad, movilidad y momento de aplicación), además de poder disminuir efectos toxicológicos y ambientales.

El método quimico posee la innegable ventaja de su eficacia, rapidez, selectividad y baja relación costo/beneficio Ross y Lembi, 2009). No obstante, el uso masivo, predominante y sin criterios técnicos-científicos de los herbicidas propició el alerta y preocupación de la sociedad en general, y científica en particular, por el desarrollo de biotipos tolerantes y resistentes a herbicidas (Powles y Yu, 2010) y de posibles efectos toxicológicos y ambientales derivados de su uso.

Resistencia a Herbicidas

La resistencia a herbicidas se define como la capacidad natural y heredable que un individuo de una especie de mala hierba posee para sobrevivir, completar su ciclo de vida y reproducirse cuando el herbicida es aplicado a las dosis recomendadas de campo (Beckie, 2006). La resistencia es un proceso evolutivo, en el que una población cambia de ser susceptible a ser resistente. *Las plantas individuales no pasan de ser susceptibles a ser resistentes, sino que es la proporción de individuos originalmente resistentes dentro de la población, la que se incrementa a lo largo del tiempo.*

El término tolerancia se usa no sólo para referirse a variaciones entre especies, sino también en relación con la variabilidad dentro de una especie (LeBaron y Gressel, 1982). En este caso, tolerancia y resistencia son expresiones que denotan diferencias en intensidad de un mismo fenómeno, considerándose la resistencia como un caso extremo y menos frecuente de tolerancia (Holt y LeBaron, 1990), o considerando la tolerancia como un mecanismo poligénico, y la resistencia como uno monogénico (Gressel, 1985).

El origen monogénico de la resistencia considera que puede estar presente un gen o un grupo de genes que otorgan resistencia debido a mutaciones aleatorias, que pueden haber ocurrido antes de la introducción del herbicida. En cualquier caso, el herbicida mata a la mayoría de las plantas susceptibles, pero los individuos resistentes sobreviven y se reproducen. En este contexto, la proporción de individuos resistentes en la población se incrementa gradualmente, hasta el punto en el que se produce un fallo en la efectividad del herbicida, considerándose esta situación cuando se observa entre un 10 y un 20% de plantas que no mueren por la aplicación. Es importante considerar que la proporción de genes resistentes en toda la población puede haberse incrementado durante años, antes de que se advierta un problema de control en campo. El grado de

resistencia en la población depende de la proporción entre individuos resistentes y susceptibles (Moss, 2002).

El conocimiento de los procesos fisiológicos responsables de la resistencia a herbicidas en las malezas, es fundamental para el diseño de una estrategia de control. Dependiendo del tipo de mecanismo de resistencia detectado, la mala hierba presentará un patrón específico en su resistencia a herbicidas, que podrá variar desde un alto grado de resistencia a determinados compuestos de una misma familia química, a una moderada resistencia a un amplio espectro de herbicidas.

La resistencia poligénica considera que la selección puede, mediante un proceso bastante menos conocido, actuar sobre la variación continua o cuantitativa, adquiriendo un incremento gradual y progresivo en la resistencia a lo largo de varias generaciones. Estas variaciones cuantitativas pueden ser causadas por un cierto número de poligenes, cada uno de los cuales, si bien produce un efecto mínimo, tiene la posibilidad de generar un nuevo rasgo en el fenotipo. De acuerdo a este segundo método, la selección puede estar actuando sobre los genes que producen resistencia, aunque sea muy leve la ventaja que aportan a la planta. El término de variación cuantitativa implica que existe un continuo de respuestas al herbicida dentro de la población, las cuales van desde susceptible, parcialmente resistente hasta altamente resistente. Esto ocurre debido a un incremento progresivo en el nivel de resistencia en toda la población, y no a un incremento en la proporción de individuos resistentes (Moss, 2002).

Mecanismos de Resistencia a Herbicidas

El conocimiento de los procesos fisiológicos responsables de la resistencia a herbicidas en las malezas, es fundamental para el diseño de una estrategia de control. Dependiendo del tipo de mecanismo de resistencia detectado, la mala hierba presentará un patrón específico en su resistencia a herbicidas, que podrá variar desde un alto grado de resistencia a determinados compuestos de una misma familia química, a una moderada resistencia a un amplio espectro de herbicidas. Asimismo, el conocimiento de estos mecanismos permite prever la posible respuesta de la población resistente al conjunto de métodos (químicos, mecánicos, culturales) seleccionados para su control, la efectividad a corto y largo plazo de los mismos y la posible aparición de nuevos problemas.

La resistencia a herbicidas involucra mecanismos dentro del sitio de acción (TS:target-site) y fuera del sitio de acción (NTS:non target-site). De acuerdo con Heap (2014), la resistencia a herbicidas es debida a cinco mecanismos primarios (Figura 3).

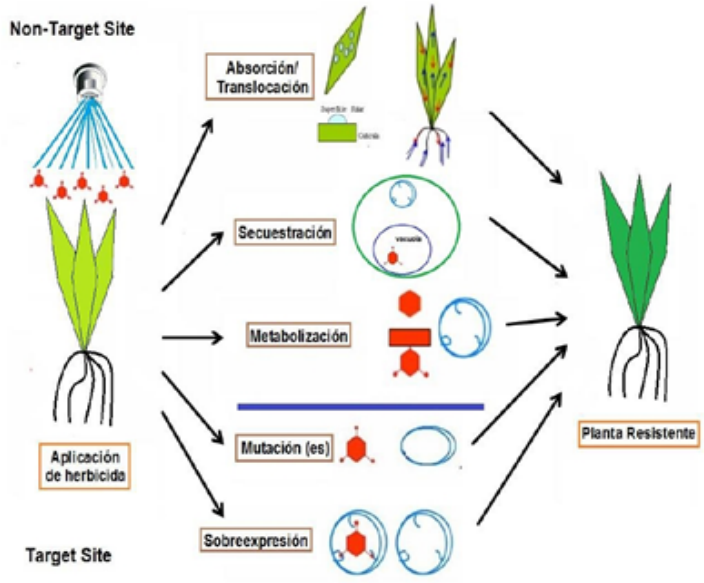


Figura 3. Mecanismos de resistencia a herbicidas dentro y fuera del sitio de acción.

Las plantas resistentes pueden expresar uno o múltiples mecanismos de resistencia al mismo o a distintos herbicidas.

1. Disminución de la absorción y/o translocación: es la restricción de movimiento de un herbicida en cantidad suficiente al sitio de acción que permite la planta para sobrevivir. Es básicamente un mecanismo de tolerancia existente en numerosos cultivos y algunas malezas (Hess,1985; De Prado *et al.*, 2001; Michitte *et al.*, 2004; Ruiz-Santaella *et al.*, 2004; Cruz-Hipolito *et al.*, 2009).

2. Secuestro de un herbicida en las paredes celulares o en vacuolas; este mecanismo reduce la concentración de herbicida que debe alcanza el sitio de acción. Son mecanismos de resistencia o tolerancia poco conocidos, pues las evidencias que los apoyan son en muchos casos circunstanciales (Coupland, 1991; Owen y Pallutt, 1991).

3. Metabolismo mejorado es el aumento de la capacidad de una planta para metabolizar (degradar) a sustancias no toxicas un herbicida antes de que la mate. La velocidad de degradación enzimática puede variar debido a factores endógenos y exógenos como el estadio de crecimiento de la planta o las condiciones climáticas, entre otros.

4. La resistencia en el target-site (a menudo una enzima) resulta por sustitución/es de

nucleótidos en la secuencia de ADN de la enzima target, causando mutaciones y alterando su traducción a proteínas, pero sin que esta pierda su función, limitando o reduciendo la capacidad de un herbicida de unirse al sitio de acción (Devine y Shimabukuro, 1994; Grownwald, 1994). Este tipo de mecanismo es el exhibido en la mayoría de los biotipos resistentes descritos hasta el momento y se caracteriza por conferir un alto grado de resistencia al herbicida empleado e incluso a otras moléculas pertenecientes a la misma familia química.

5. La amplificación génica/sobreexpresión aumenta la producción de la enzima diana (target-site), por lo que se requiere una concentración más alta de herbicida para para inhibir la enzima diana y causar la muerte.

Además de la resistencia monoherbicida, las malas hierbas pueden desarrollar resistencia cruzada o resistencia múltiple.

La resistencia cruzada ocurre cuando un único mecanismo de resistencia confiere resistencia a más de un herbicida. La resistencia cruzada de target-site es el tipo más común, y es el resultado de un sitio diana alterada que confiere resistencia a otros herbicidas que inhiben la misma enzima (Heap, 2014).

La resistencia múltiple se presenta cuando una planta expresa más de un mecanismo de resistencia, y es generalmente el resultado de la selección secuencial de mecanismos de resistencia a herbicidas con diferentes sitios de acción o por medio de la acumulación de genes de resistencia a través de flujo de polen (Heap y Le Barron, 2001). La resistencia a herbicidas múltiples dentro de biotipos individuales está muy extendida (Harker y O´Donovan, 2013).

Problemática de la Resistencia a Nivel Mundial

No existe una clara relación entre la familia o el género botanico y su tendencia a desarrollar resistencia. La aparición de malas hierbas resistentes a herbicidas no tuvo lugar hasta finales de la década de 1960, con poblaciones de *Senecio vulgaris* resistentes a las s-triazinas, atrazina y simazina en un vivero forestal.

Desde la identificación del primer biotipo resistente, Heap (2018) que el incremento anual de malezas resistentes a herbicidas experimentó un crecimiento exponencial a partir de la década de 1990 (Figura 4).

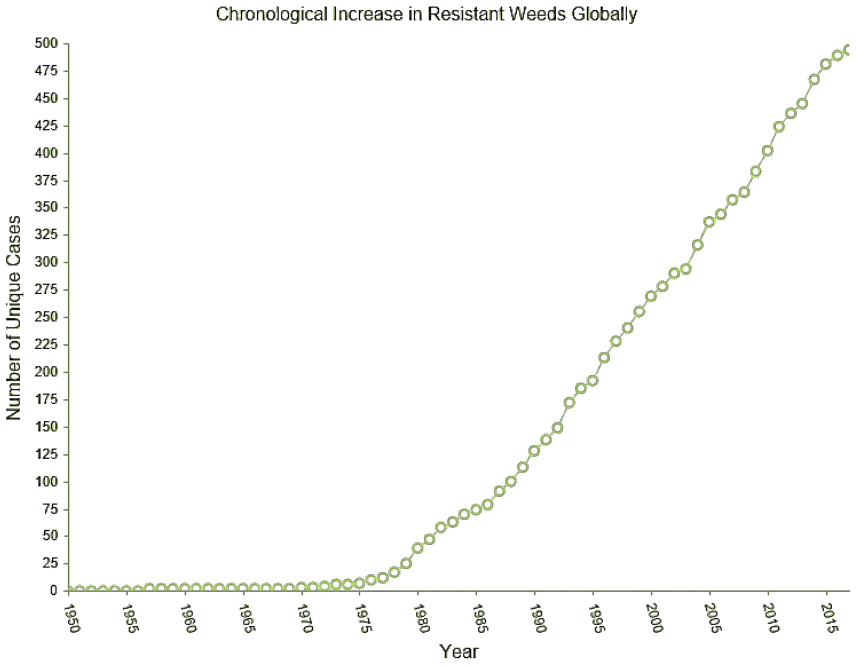


Figura 4. Incremento del número de malezas resistentes a herbicidas hasta el año 2017 (Heap, 2018).

Las gramíneas representan el 25% de las peores malezas del mundo y constituyen el 33% de todas las especies resistentes y el 40% de todos los biotipos resistentes (Moss, 2002; Beckie, 2006).

Actualmente se han registrado 494 casos únicos de resistencia, en 256 especies dicotiledóneas y 238 monocotiledóneas) en 91 cultivos en 67 países (Heap, 2018).
Las familias Poaceas y Asteraceae comprenden especies con gran relevancia como invasoras de cultivos, alcanzando valores de 31% y 16% del total de todas las especies resistentes a herbicidas, respectivamente (Figura 5).

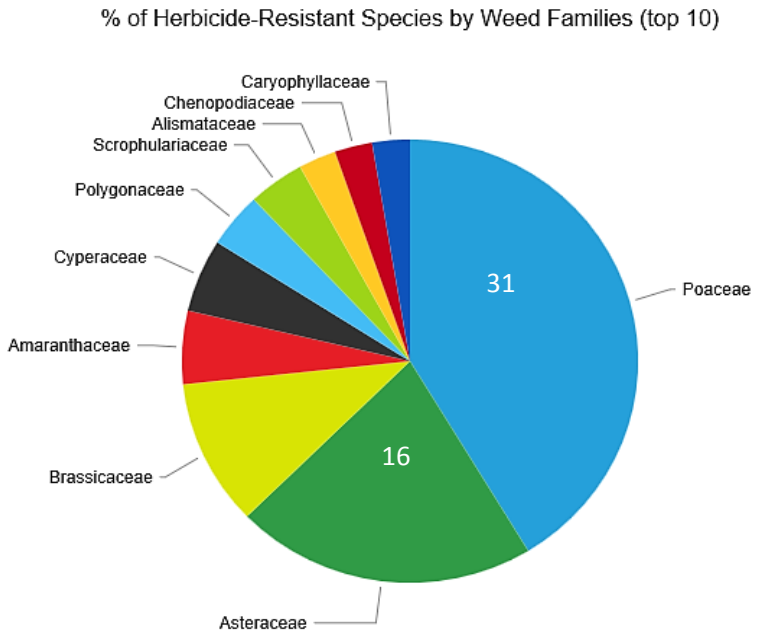


Figura 5. Resistencia a herbicidas por familia botánica (Heap, 2018).

Un problema importante es el rápido y continuo desarrollo de malezas resistentes a herbicidas inhibidores de las enzimas ALS, ACCasa y especialmente los inhibidores de la EPSPS, mostrando estos herbicidas en los ultimos años una tasa alta de crecimiento de eventos confirmados en el mundo. Estos herbicidas al ser productos selectivos y de uso predominante en los sistemas agrícolas ejercen gran presión de selección sobre las malezas.

Actualmente, además de los inhibidores de las enzimas ALS, ACCasa y EPSPS, los grupos de herbicidas más importantes que se ven afectados por la problemática de la resistencia son los inhibidores del fotosistema I y II, e inhibidores de las auxinas sintéticas (Heap, 2018; Mallory-Smith *et al.*1990). Estos grupos químicos comprenden a los principios activos más usados en todos los cultivos de producción extensiva e intensiva del mundo (Figura 6).

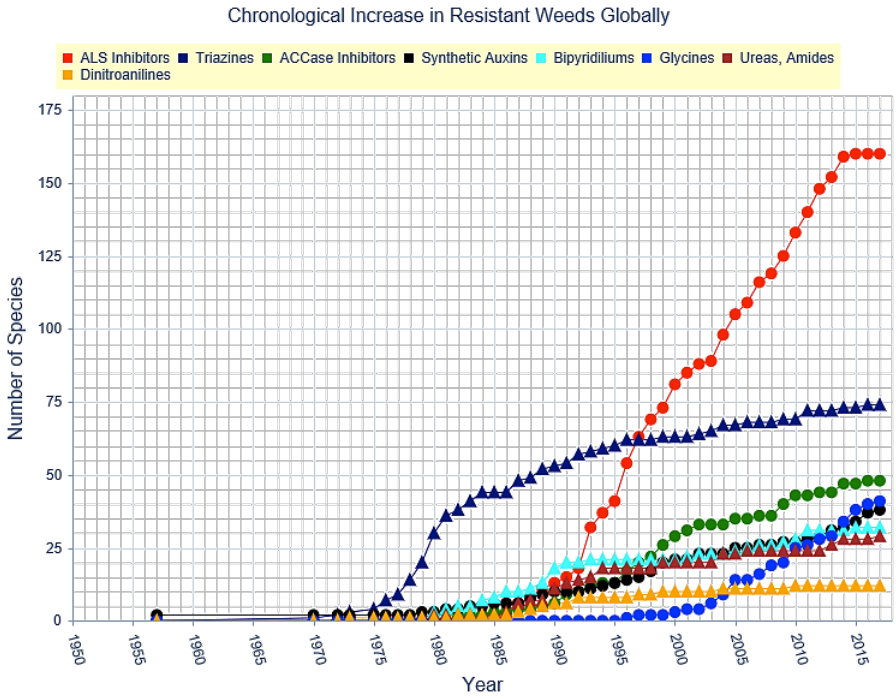
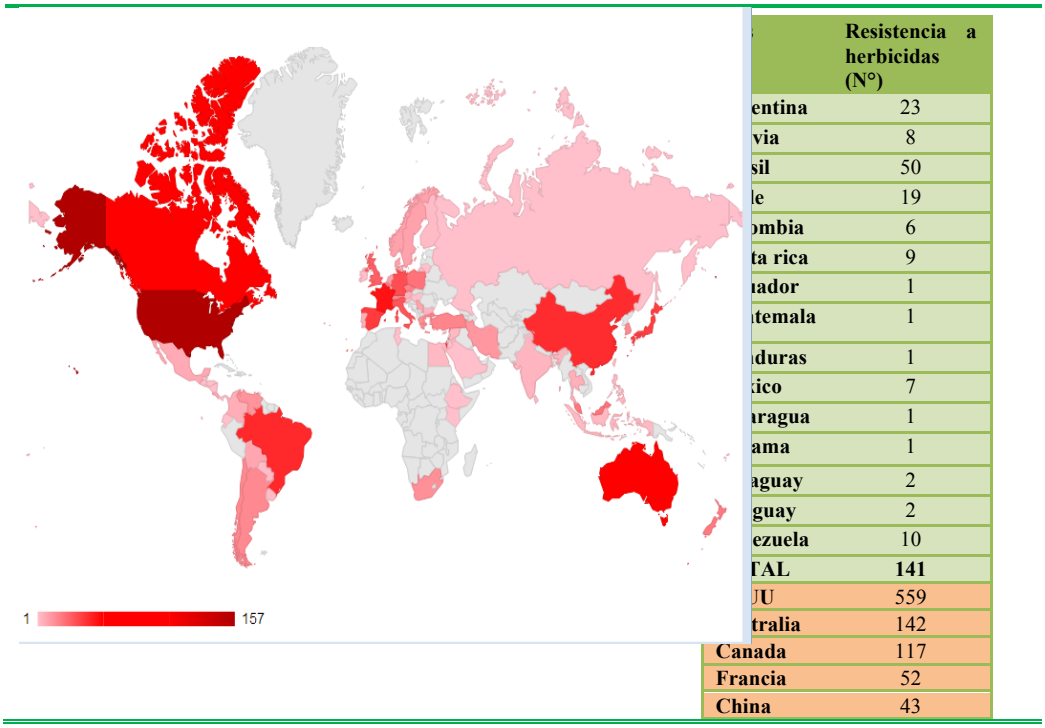


Figura 6. Evolución histórica del número de biotipos resistentes a distintas familias químicas de herbicidas (Heap, 2018).

Este aumento en biotipos continúa amenazando a muchas regiones agrícolas del mundo, sobre todo en aquellas en donde existen casos de malezas que presentan resistencia múltiple. La especie más problemática en todo el mundo es *Lolium rigidum*, que ha sido identificada como resistente en 12 países, ha desarrollado resistencia a 11 sitios de acción en mas de 20 cultivos diferentes. *Conyza canadensis, Avena fatua, Amaranthus tuberculatus, Echinochloa crus-galli y Chenopodium álbum* constituyen las segundas malezas resistentes a herbicidas más importantes a nivel mundial.

La resistencia a herbicidas observada en Latinoamérica esta generalmente asociada con una alta presión de selección, y está desarrollada sobre dos mecanismos de resistencia, la reducción de la absorción y/o traslocación del herbicida y posterior pérdida de transporte vía xilema/floema del herbicida a la proteína de enlace, conocidos como NTSR y la pérdida de afinidad entre la proteína de enlace y el herbicida o bien por una sobreexpresión de esa proteína llamada TSR) (González-Torralva *et al.,* 2014). Desde la comercialización del glifosato hasta la primera confirmación de resistencia transcurrio 20 años (Powles *et al*., 1998).

La resistencia en Latinoamérica muestra que existen diferencias significativas en el número de casos de resistencia (malezas/herbicida) en relación a los reportados en los países desarrollados (Figura 7).



	Resistencia a herbicidas (N°)
entina	23
via	8
sil	50
le	19
ombia	6
ta rica	9
ador	1
temala	1
duras	1
ico	7
ragua	1
ama	1
aguay	2
guay	2
ezuela	10
TAL	**141**
JU	559
tralia	142
Canada	117
Francia	52
China	43

Figura 7. Número de casos de resistencia a herbicidas en Latino América en relación a valores reportados en países desarrollados (Heap, 2018).

La distribución relativa de resistencia basados en el modo de acción de los herbicidas muestran que los tres grupos más importantes (ALS, triazinas y ACCasa) comprenden 74% y 65% de los casos de resistencia en los países desarrollados y en desarrollo, respectivamente. En ambos grupos de países, la resistencia a las triazinas continúa siendo la más frecuente de acuerdo con el número de biotipos. En los países desarrollados, la resistencia al grupo ALS tiene una frecuencia equivalente al doble de la resistencia a ACCasa. En los países en desarrollo, la frecuencia de resistencia de ambos modos de acción es prácticamente la misma. Los herbicidas derivados de los bipiridilos, auxinas sinteticas, ureas y amidas sustituidas, contribuyen con más casos de resistencia en los países en desarrollo que en los países industrializados.

Los mayores problemas encontrados en cereales (arroz, trigo, maíz) están asociados con la competencia de malezas gramíneas (*Echinochloa spp, Lolium spp, Phalaris spp, Urochloa spp., sorghum sp.* y otras), que son de difícil manejo y control. Asociado a

esto, se encuentra la resistencia conferida a herbicidas con distintos modos de acción (Valverde, 2007).

Resistencia a Glifosato

Actualmente el herbicida glifosato, correspondiente a los inhibidores de la enzima EPSPS (5-enolpiruvilshikimato-3-fosfato sintasa) constituye el principio activo con mayor comercialización y uso en todos los sistemas agrícolas del mundo.

El glifosato [N-(fosfonometil) glicina] es un herbicida posemergente de amplio espectro (Duke y Powles, 2008). Actúa inhibiendo la 5-enolpiruvilshiquimato-3-fosfato sintasa (EPSPS), responsable de la biosíntesis del corismato, un intermediario en la vía del ácido shiquímico que guía la síntesis de aminoácidos aromáticos esenciales (triptófano, fenilalanina y tirosina) de las plantas (Franz *et al*., 1997) (Figura 8).

Desde su introducción al mercado (1974), el glifosato se ha convertido en el herbicida más popular y de mayor venta global para el control de malas hierbas (Orcaray *et al.,* 2012, Székács y Darvas, 2012; Duke y Powles, 2008). La dependencia casi exclusiva de glifosato para el control de malezas ha llevado a la evolución de las poblaciones de malas hierbas tolerantes y resistentes, influenciado principalmente, pero no exclusivamente, por la adopción de cultivos transgénicos (Sammons y Gaines, 2014; Yanniccari *et al.,*2016).

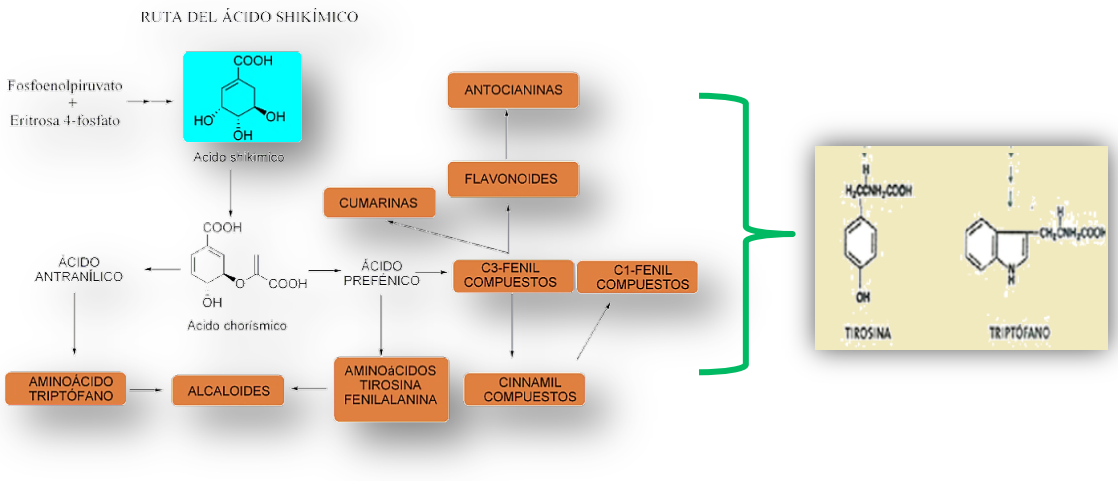


Figura 8. Biosíntesis de aminoácidos aromáticos vía ácido shiquímico.

Las primeras malezas resistentes al glifosato fueron *Lolium rigidum* en Australia (Prately *et al*., 1999; Powles *et al.,*1998) y *Eleusine indica* en Malaysia (Lee y Ngim, 2000). En 1996, *Conyza Canadensis* fue el primer caso de maleza resistente al glifosato que apareció en un cultivo de soja tolerante al glifosato en Delaware y en Tennessee,

EE. UU. (Van Gessel, 2001; Duke y Powles, 2008) como consecuencia del uso reiterado de glifosato sin un programa de manejo integrado de malezas.

Actualmente existen 41 especies resistentes a glifosato en el mundo con 299 casos reportados (Heap., 2018). De estas malezas, las más significantes, en términos económicos son *Palmer amaranth y Conyza canadensis*. Otras malezas resistentes al glifosato que son potencialmente graves son *Ambrosia artemisifolia* y *Sorghum halepense.*

Resistencia a Glifosato en Latinoamérica

En Latinoamérica, desde hace casi 40 años las poblaciones de malas hierbas están bajo presión de selección continua por el uso de glifosato, constituyendo actualmente como el herbicida con mayor importancia para el control de malas hierbas en esta región (Sammons y Gaines, 2014). Esta presión de selección propicio la aparición de 44 casos confirmados de resistencia, lo que representa un 14.7 % de casos en relación al total de eventos confirmados con otras familias químicas (Tabla 1).

México muestra escasa información sobre estudios de distribución de malas hierbas resistentes a herbicidas y sus implicaciones, ya sean introducidas o nativas."The International Survey of Herbicide Resistant Weeds" declara que en México existen siete casos de malas hierbas resistentes a herbicidas (*Phalaris minor Retz, Phalaris paradoxa* L*, Avena fatua* L.*, Sorghum halepense (*L.*) Pers, Leptochloa virgata* (L.) *P.Beauv., Bidens pilosa* L.*, Ixophorus unisetus* (J.Presl) Schltdl, correspondiendo dos de ellas a resistencia a glifosato (Heap, 2018).

Tabla 1. Relación de malezas resistentes y grupos químicos de los herbicidas confirmada en Latinoamérica, 2018.

Pais	Especie	Año	Mec. Resistencias (N°)
Argentina	*Amaranthus hybridus (syn: quitensis; Sorghum halepense; Lolium perenne ssp. Multiflorum; Cynodon hirsutus; Raphanus sativus; Echinochloa colona; Avena fatua; Eleusine indica; Digitaria insularis; Amaranthus palmeri. Amaranthus hybridus. Hirschfeldia incana* .	1996-2016	ALS inhibitors B/2 (3) EPSP synthase inhibitors G/9 (10) ACCase inhibitors A/1 (3) Synthetic Auxins (O/4) (1) Resistencia Multiple: (3) ALS inhibitors B/2-EPSP synthase inhibitors G/9 (2) ; ACCase inhibitors A/1-EPSP synthase inhibitors G/9 /1). ALS inhibitors (B/2)-Synthetic Auxins (O/4)(1) EPSP synthase inhibitors (G/9)-Synthetic Auxins (O/4) (1)

Bolivia	*Eriochloa punctata; Sorghum bicolor ssp. drummondii (=Sorghum sudanese); Amaranthus hybridus (syn: quitensis);Eleusine indica; Echinochloa colona*.	1997-2007.	ALS inhibitors B/2 (2) EPSP synthase inhibitors G/9 (1) ACCase inhibitors A/1 (4) PPO inhibitors E/14 (1).
Brasil	*Bidens pilosa; Euphorbia heterophylla; Bidens subalternans Urochloa plantaginea (=Brachiaria plantaginea); Sagittaria montevidensis; Echinochloa crus-pavonis; Echinochloa crus-galli var. crus-gall; Cyperus difformis; Fimbristylis miliacea; Raphanus sativus; Digitaria ciliaris; Lolium perenne ssp. multiflorum; Eleusine indica; Parthenium hysterophorus; Conyza bonariensis; Conyza canadensis; Oryza sativa var. Digitaria insularis; Conyza sumatrensis; Avena fatua; Amaranthus retroflexus; Amaranthus viridis; Raphanus raphanistrum; Ageratum conyzoides; Chloris elata; Cyperus iria; Amaranthus palmeri;*	1993-2017	ALS inhibitors B/2 (15) EPSP synthase inhibitors G/9 (7) ACCase inhibitors A/1 (5) Synthetic Auxins O/4 (2) PPO inhibitors E/14 (2) Resistencia Multiple: () ALS inhibitors B/2-EPSP synthase inhibitors G/9 (3) ; ACCase inhibitors A/1-EPSP synthase inhibitors G/9 (2). ALS inhibitors B/2-PPO inhibitors E/14 (1); ALSinhibitorsB/2-PhotosystemII inhibitors (1/5 (4); ALSinhibitorsB/2-Synthetic Auxins O/4 (1); ALS inhibitors B/2 PSII inhibitors (Nitriles) C3/6 (1); ACCase inhibitors A/1-ALS inhibitors B/2 (1). ALS inhibitors (B/2)-PSI Electron Diverter (D/22)-EPSP synthase inhibitors (G/9). PSII inhibitor (Ureas and amides) (C2/7)-PSI Electron Diverter (D/22)- PPO inhibitors (E/14)-EPSP synthase inhibitors (G/9)-Synthetic Auxins (O/4).
Chile	*Lolium rigidum; Avena fatua; Lolium perenne ssp. multiflorum; Cynosurus echinatus; Lolium perenne; Alisma plantago-aquatica;Schoenoplectus mucronatus(=Scirpus mucronatus);Sorghum halepense; Raphanus sativus; Anthemis cotula; Anthemis arvensis; Silene gallica.*	1997-2012.	ALS inhibitors B/2 (7) EPSP synthase inhibitors G/9 (1) ACCase inhibitors A/1 (5) Resistencia multiple ALS inhibitors B/2-EPSP synthase inhibitors G/9 (1); ACCase inhibitors A/1- ALS inhibitors B/2 (3); ACCase inhibitors A/1-EPSP synthase inhibitors G/9 (1); ACCase inhibitors A/1-ALS inhibitors B/2- EPSP synthase inhibitors G/9 (1).
Colombia	*Echinochloa colona; Ischaemum rugosum;Parthenium hysterophorus;Conyza bonariensis; Eleusine indica*	1988-2006	Synthetic Auxins O/4 (1); ACCase inhibitors A/1 (1); PSII inhibitor (Ureas and amides) C2/7 (1); EPSP synthase inhibitors G/9 (3).
Costa Rica	*Echinochloa colona; Ixophorus unisetus; Eleusine indica; Oryza sativa var. sylvatica, Paspalum paniculatum*	1987-2013.	ALS inhibitors B/2 (3); ACCase inhibitors A/1(1); EPSP synthase inhibitors G/9 (2);PSII inhibitor (Ureas and amides) C2/7 (1); Resistencia Multiple: ACCase inhibitors A/1-ALS inhibitors B/2-PSII inhibitor Ureas and amides C2/7 (1).

Ecuador	*Euphorbia heterophylla*	1990	PSII inhibitor (Ureas and amides) C2/7 (1)
El Salv.	*Echinochloa colona*	1999	PSII inhibitor (Ureas and amides) C2/7 (1)
Guatemala	*Echinochloa colona*	1999	PSII inhibitor (Ureas and amides) C2/7 (1)
Honduras	*Echinochloa colona*	1999	PSII inhibitor (Ureas and amides) C2/7 (1)
Mexico	*Phalaris minor; Phalaris paradoxa; Avena fatua; Sorghum halepense; Leptochloa virgata; Bidens pilosa; Ixophorus unisetus.*	1996-2014.	ACCase inhibitors A/1 (3); ALS inhibitors B/2 (2); EPSP synthase inhibitors G/9 (2).
Nicaragua	*Echinochloa colona*	2000	ACCase inhibitors A/1 (1).
Panama	*Echinochloa colona*	1999	PSII inhibitor (Ureas and amides) C2/7 (1)
Paraguay	*Euphorbia heterophylla; Digitaria insularis,*	1995-2005.	ALS inhibitors B/2 (1); EPSP synthase inhibitors G/9 (1).
Uruguay	*Echinochloa crus-galli var. crus-galli.*	2013	Synthetic Auxins O/4 (1); ALS inhibitors B/2 (1).
Venezuela	*Echinochloa colona; Ischaemum rugosum;Rottboellia cochinchinensis (=R. exaltata); Fimbristylis miliacea; Sorghum halepense; Leptochloa scabra; Cyperus odoratus; Cyperus iria.*	2000-2015	PSII inhibitor (Ureas and amides) C2/7 (2); ALS inhibitors B/2 (5); Resistencia Multiple ACCase inhibitors A/1-ALS inhibitors B/2-PSII inhibitor Ureas and amides (2); ACCase inhibitors A/1-EPSP synthase inhibitors G/9 (1).

En Centro América y el Caribe, se han confirmado muy pocos eventos de resistencia, alcanzando cinco países y seis especies (Heap, 2018). En Costa Rica están confirmados dos casos de resistencia a glifosato, en Paspalum paniculatum y en Eleusine indica. En la República Dominicana, estudios recientes detectaron resistencia a glifosato en biotipos de Parthenium hysterophorus (Jimenez et al., 2014) y Phaseolus lathyroides con tolerancia natural. En Sudamérica, la producción frutícola de exportación de manzana, vid, limón, naranja y café en Argentina, Brasil, Chile y Colombia representan una de las principales actividades económicas en el contexto regional. El control de malezas en las huertas es realizado mediante el uso de varias aplicaciones por año de glifosato. Por ello, los primeros informes de resistencia al glifosato involucraron estos sistemas productivos, incluyendo poblaciones de taxones muy diversos como *Lolium multiflorum* (Lam.) (Chile, Argentina y Brasi); *Conyza bonariensis* L., (Argentina y Brasil); *Conyza canadensis* L. (Brasil); *Parthenium hysterophorus* L. (Colombia). Además, la aparición de *Sorghum halepense* L. y *Euphorbia heterophylla* L. resistente a glifosato en campos de soja resistentes al glifosato de Argentina y Brasil acrecentó la

magnitud de esta problemática (Vila-Aiub, 2008; Vidal *et al*., 2007; Powles y Shaner, 2001; Perez-Jones *et al*., 2007).

Brasil actualmente tiene declarados 50 casos de resistencia a herbicidas correspondientes a 29 biotipos resistentes encontrados en cultivos de soja GR (resistente al glifosato), arroz, maíz, frutales y otros. Las especies confirmadas son: *Ageratum conyzoides, Amaranthus palmeri, Amaranthus retroflexus, Amaranthus viridis, Avena fatua, Bidens pilosa, Bidens subalternans, Chloris elata, Conyza bonariensis, Conyza canadensis, Conyza, sumatrensis, Cyperus difformis, Cyperus iria, Digitaria ciliaris, Digitaria insularis, Echinochloa crus-galli var. crus-galli, Echinochloa crus-pavonis, Echium plantagineum, Eleusine indica, Euphorbia heterophylla, Fimbristylis miliacea, Lolium perenne sp. multiflorum, Oryza sativa var. sylvatica, Parthenium hysterophorus, Raphanus raphanistrum, Raphanus sativus, Sagittaria montevidensis*, *Urochloa plantaginea* (=Brachiaria plantaginea) (Heap, 2018). Entre ellas, este país reporto 15 eventos confirmados de resistencia directa y múltiple a glifosato.

En Argentina, en los últimos 20 años se han producido cambios importantes en las poblaciones de malezas en los diferentes sistemas de producción agrícola. Algunas de las causas serían la extensa superficie sembrada con soja GR, la gran difusión de la siembra directa, el uso masivo y único del herbicida glifosato, la escasa rotación de cultivos y de principios activos con diferentes modos de acción, la ocupación de tierras menos apta para la agricultura y el intenso desmonte. Esta situación generó una nueva problemática como es la aparición de malezas que se relacionan con las prácticas mencionadas, surgiendo por un lado, las especies que mejor se adaptan a la labranza cero, y por otro, la aparición de biotipos con tolerancia o resistencia a los diferentes herbicidas de uso frecuente en estos sistemas, como por ejemplo el glifosato.

Actualmente el Servicio Nacional de Sanidad y Calidad Agroalimentaria (Senasa, 2018) dio a conocer el listado de 23 especies de malezas cuya resistencia a distintos principios activos de herbicidas fue confirmada (Tabla 2), entre ellas están confirmadas casos de resistencia a glifosato en especies: *Amaranthus palmeri*, *Amaranthus hybridus*, *Conyza bonariensis*, *Cynodon hirsutus*, *Digitaria insularis*, *Echinochloa colona*, *Eleusine indica* ,*Lolium perenne* sp.*multiflorum*, *Sorghum halepense, Amaranthus hybridus* (*Syn.quitensis*) e *Hirschfeldia incana*. Esta problemática no es una consecuencia directa del uso de glifosato, sino del uso sin criterio técnico que se hace de él en Argentina.

Esta práctica ejerció una severa presión de selección de malezas y su consecuencia fue y seguirá siendo la difusión de aquellas más adaptadas a los sistemas de producción agrícola modernos (Rainero, 2008).

Actualmente, en el resto de los países de Sudamérica se reportó menor número de eventos de resistencia a glifosato, entre ellos Bolivia, confirmó un caso, Chile, cuatro casos, Colombia, tres casos, y Venezuela y Paraguay un caso cada uno.
En Latinoamerica se destacan las especies resistentes a herbicidas pertenecientes a las familias Poaceae y Asteraceae, alcanzando valores de 61.5% y 11.5 %, respectivamente, en relación al número total por familia botánica. Entre ellas, los biotipos que han incrementado en forma significativa su presencia en las regiones con cultivos de citrus y de producción extensiva como soja y maíz del Caribe y Sudamérica, respectivamente, son las correspondientes a las especies *Parthenium hysterophorus* L y *Chloris spp.*

Parthenium hysterophorus L., el parthenium o partenio, es una hierba anual de la familia Asteraceae que es nativa del Golfo de México y otros países de América Latina (Rosario *et al.,* 2013). Posee prolífica producción de semilla (130.000 a 200.000 semillas m^{-2}), y la capacidad de las semillas para persistir en el suelo y germinar en un amplio rango de temperaturas, han contribuido a la distribución generalizada del partenio en cultivos perennes y anuales (huertos, cítricos, soja, maíz) así como en las áreas circundantes (Joshi, 1991; Pandey y Saini, 2002; Navie, 2003; Adkins y Shabbir, 2014). El clima subtropical de las islas del Caribe, como Cuba y República Dominicana, permite la germinación, el crecimiento y la reproducción durante todo el año del partenio, lo que contribuye a su amplia distribución en la región. El glifosato se ha usado repetidamente en áreas de cultivos perennes y campos en barbecho en las islas del Caribe durante muchos años para manejar el partenio y otras malezas problemáticas. Sin embargo, los productores han observado recientemente un control reducido de partenio con aplicaciones de glifosato únicas o múltiples. Informes previos han documentado partenio resistente al glifosato en Colombia (Rosario *et al*., 2013), Florida (EE. UU.) (Fernández, 2013) y República Dominicana (Jiménez *et al*., 2014), pero en estos tres casos las causas de la resistencia al glifosato no ha sido concluyente.

El género *Chloris* ha sido tratado en un sentido amplio, incluyéndose especies de *Eustachys* y *Trichloris* Benth, aunque en la actualidad existe coincidencia de mantener

los tres géneros independientes. Esta disparidad de conceptos se basa en la confusa nomenclatura de las entidades en cuestión, por lo que una de las dificultades que se presenta al abordar el estudio de las especies pertenecientes a este género fue su caracterización.

Este género fue descrito por Peter Olof Swartz en 1788, comprendiendo al menos 60 especies en Latinoamérica propias de regiones tropicales, subtropicales y cálidas de ambos hemisferios a las que habría que sumar las correspondientes al género *Trichloris* y otras especies pertenecientes a la tribu de las Chlorideas (Barkworth, 2007).

Son plantas C_4 de desarrollo estival, que viven principalmente en zonas semiáridas (Anderson, 1974; Watson y Dallwitz, 1992; Molina y Agrasar, 2004).

En América, estas especies se distribuyen desde regiones tropicales y subtropicales hasta regiones templadas de Uruguay y Argentina (Anderson, 1974; Nicora y Rúgolo, 1987). La mayoría de las especies son perennes y cespitosas, con rizomas breves, no obstante algunos taxones son estoloníferos. Las especies anuales no son numerosas.

El género *Chloris* posee un cierto nivel de tolerancia natural al glifosato en comparación con especies de otros géneros. Esto permite que las plantas sobrevivan y se reproduzcan después de aplicaciones de herbicidas de campo letales para las plantas silvestres de otras especies.

En Cuba, los cítricos constituyeron uno de los principales rubros económicos del país. Actualmente este cultivo presenta una compleja situación, dada por las dificultades económicas, climáticas, y la presencia de enfermedades y malezas que constituyen una amenaza para las plantaciones (EcuRed, 2017). Los estudios de malezas resistentes a herbicidas en Cuba son escasos debido a la falta de conocimiento del problema. Esta situación es similar a lo observado en República Dominicana, en el que los estudios de evaluación ya comenzaron a desarrollarse (Bracamonte *et al*., 2016).

Los productores de cítricos de este país observan en sus plantaciones individuos de este género que no están siendo controladas con las dosis de campo recomendadas de glifosato (720 g ae ha^{-1}). Debido que las estrategias de control de malezas en campos comerciales se realiza principalmente con el uso de glifosato, la identidad taxonómica de las especies involucradas es un requisito previo para establecer estrategias de malezas eficientes. Esto es particularmente importante en el caso del género *Chloris* donde las especies presentan dificultad para su identificación debido a la complejidad taxonómica y de nomenclatura existente (Molina y Agrasar, 2004).

Actualmente México es el segundo mayor productor y principal exportador de Lima persa *(Citrus latifolia Tan.)* (USDA, 2017). El manejo de cultivos de citrus es mecánico, químico (principalmente a base de glifosato) y con métodos combinados. En este país, las especies de *Chloris* son malas hierbas invasoras que se encuentran en áreas alteradas y conservadas. Entre ellas, biotipos de *Chloris barbata* fueron observados sobreviviendo a la dosis estándar ampliamente utilizada de 720 g ae ha^{-1}de glifosato. La pérdida de susceptibilidad al glifosato en *C. barbata* puede deberse a un aumento en su tolerancia innata o al desarrollo de mecanismos de resistencia.

En Argentina, entre las especies que han incrementado en forma significativa su presencia en el área núcleo agrícola y particularmente en la región centro y norte del país son las correspondientes al género *Chloris* (Figura 9). Entre ellas, *Chloris virgata Sw* y *Chloris elata* Desv. son especies adaptadas a suelos no modificados. Esta situación facilitó su adaptación a sistemas de siembra directa, tornándose actualmente malezas muy competitivas y de difícil control con glifosato en cultivos de soja y maíz. De las 41 especies de malas hierbas resistentes a glifosato (Heap, 2018) en todo el mundo, entre ellas se encuentra *Chloris elata* presentes en sistemas agrícolas de Brasil y Argentina (Figura 9), respectivamente.

Chloris virgata es una especie anual, presenta alta tolerancia a dosis de campo comerciales de glifosato y constituye un importante problema en cultivos de soja en la región central del país (AAPRESID-REM- 2018).

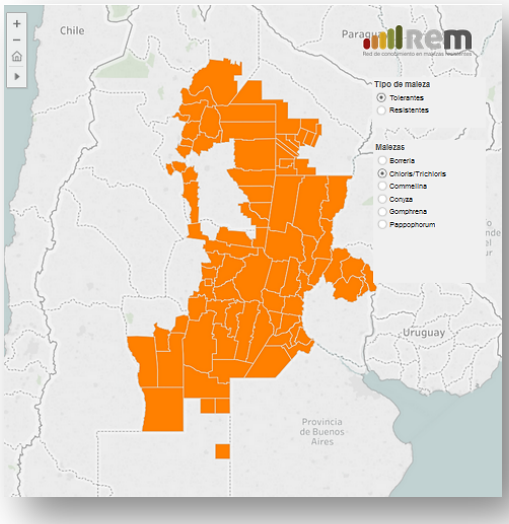


Figura 9. Área de cobertura de *Chloris spp* en la región productiva agrícola de Argentina. Aapresid- REM-, 2018.

Estas especies aumentaron considerablemente su difusión propiciada por la amplia adopción del sistema tecnológico Siembra Directa-Soja RR (Roundup Ready)-glifosato en países de amplias superficies de siembra como Brasil y Argentina. Esta tecnología propició un aumento de la presión de selección y por ello, la difusión de biotipos resistentes a este principio activo como *Chloris elata* y *Chloris virgata.* Estas especies no son homogéneas en cuanto su distribución geográfica así como tampoco en lo referente a su respuesta a herbicidas. Esta situación, además de la dificultad en la identificación botanicia de las especies complica significativamente su estudio y la posibilidad de disponer de alternativas tecnológicas eficientes y homogéneas de control.

Dependiendo del tipo de mecanismo de resistencia desarrollado, la mala hierba presentará un patrón específico en su tolerancia a herbicidas que podrá variar desde un alto grado de resistencia a determinados compuestos de una misma familia química, a una moderada resistencia a un amplio espectro de herbicidas. El conocimiento de los mecanismos de resistencia/tolerancia involucrados y los procesos bioecológicos responsables de la resistencia a herbicidas y su difusión es fundamental para el diseño de una estrategia de control eficiente (Fischer, 2008; Duke y Powles, 2009).
Actualmente, en Latinoamérica no han sido descritos los mecanismos involucrados en la resistencia/tolerancia de las especies del género *Chloris* al herbicida glifosato ni la caracterización morfológica mediante estudios de diferenciación de estas especies con dificultad para su control. Debido a la gran variedad de especies pertenecientes a este género, la caracterización morfológica y el uso de marcadores moleculares constituye una valiosa herramienta para la realización de estudios de diferenciación de especies pertenecientes a este género en base al ADN.

La caracterización morfológica mediante estudios de diferenciación de especies, la determinación del nivel de resistencia a glifosato y los mecanismos involucrados permitirá establecer estrategias de manejo alternativas, integradas y sustentables de *Parthenium hysterophorus* y de las especies de *Chloris* presentes en áreas agrícolas de Latinoamérica.

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CAPÍTULO II

Glyphosate-resistant *Parthenium hysterophorus* in the Caribbean Islands. Non Target Site Resistance and Target Site Resistance in relation to resistance levels.

Bracamonte, E., Fernández-Moreno, P. T., Barro, F., & De Prado, R. (2016). Glyphosate-resistant Parthenium hysterophorus in the Caribbean islands: non target site resistance and target site resistance in relation to resistance levels. *Frontiers in plant science*, *7*, 1845.

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ABSTRACT

Glyphosate has been the most intensely herbicide used worldwide for decades, and continues to be a single tool for controlling weeds in woody crops. However, the adoption of this herbicide in a wide range of culture systems has led to the emergence of resistant weeds. Glyphosate has been widely used primarily on citrus in the Caribbean area, but a study of resistance in the Caribbean islands of Cuba and the Dominican Republic has never been carried out. Unfortunately, *Parthenium hysterophorus* has developed glyphosate-resistance in both islands, independently. The resistance level and mechanisms of different *P. hysterophorus* accessions (three collected in Cuba (Cu-R) and four collected in the Dominican Republic (Do-R) have been studied under greenhouse and laboratory conditions. In *in vivo* assays (glyphosate dose causing 50% reduction in above-ground vegetative biomass and survival), the resistance factor levels showed susceptible accessions (Cu-S ≥ Do-S), low-resistance accessions (Cu-R3 < Do-R4), medium-resistance accessions (Do-R3<Cu-R2< Do-R2) and high-resistance accessions (Do-R1<Cu-R1). In addition, the resistance factor levels were similar to those found in the shikimic acid accumulation at 1000 μM of glyphosate (Cu-R1 ≥ Do-R1 > Do-R2 > Cu-R2 > Do-R3 > Do-R4 > Cu-R3 >> Cu-S ≥ Do-S). Glyphosate was degraded to aminomethylphosphonic acid, glyoxylate and sarcosine by >88% in resistant accessions except in Cu-R3 and Do-R4 resistant accessions (51.12 and 44.21, respectively), whereas a little glyphosate (<9.32%) was degraded in both susceptible accessions at 96 h after treatment. There were significant differences between *P. hysterophorus* accessions in the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) activity enzyme with and without different glyphosate rates. The R accessions showed values of between 0.026 and 0.21 μmol μg^{-1} TSP protein min^{-1} basal EPSPS activity values with respect to the S (0.024 and 0.025) accessions. The same trend was found in the EPSPS enzyme activity treated with glyphosate, where a higher enzyme activity inhibition (glyphosate μM) corresponded to greater resistance levels in *P. hysterophorus* accessions. One amino acid substitution was found at position 106 in EPSPS, consisting of a proline to serine change in Cu-R1, Do-R1 Do-R2. The above-mentioned results indicate that high resistance values are determined by the number of defense mechanisms (target-site and non-target-site resistance) possessed by the different *P. hysterophorus* accessions, concurrently.

RESUMEN

El glifosato ha sido el herbicida más utilizado en todo el mundo durante décadas, y actualmente continúa siendo un metodo sencillo para el control eficaz de malas hierbas en cultivos leñosos. Sin embargo, la adopción de este herbicida en una amplia gama de sistemas de cultivo ha conducido a la aparición de malas hierbas resistentes. El glifosato ha sido utilizado ampliamente en cultivos de cítricos en la zona del Caribe, pero ningún estudio de resistencia en Cuba y la República Dominicana ha sido desarrollado hasta el presente. En este contexto, *Parthenium hysterophorus* ha desarrollado resistencia al glifosato en ambos países de forma independiente. El nivel y los mecanismos de resistencia de tres poblaciones de *P. hysterophorus* colectados en Cuba (Cu-R) y cuatro en la República Dominicana (Do-R) fueron evaluadas en condiciones de invernadero y de laboratorio. Los niveles de factor de resistencia obtenidos de ensayos in vivo (GR_{50} y DL_{50}), mostraron las poblaciones susceptibles Cu-S≥ Do-S, poblaciones con baja resistencia (Cu-R3 <Do-R4), poblaciones con resistencia media (Do-R3 <Cu-R2 <Do-R2) y poblaciones con alta resistencia (Do-R1<Cu-R1). Los niveles de los factores de resistencia fueron similares a los encontrados en la acumulación de ácido shikímico a 1000 μM de glifosato (Cu-R1≥Do-R1> Do-R2> Cu-R2> Do-R3> Do-R4> Cu-R3 >>CU-S≥Do-S). Los resultados mostraron que glifosato se degrada en >88% a ácido aminometilfosfónico (AMPA), sarcosina y glioxilato en las poblaciones resistentes, excepto en Cu-R3 y Do-R4 (51.12 y 44.21, respectivamente), mientras que poco glifosato (<9,32%) se degrada en ambas poblaciones susceptibles a las 96 h después del tratamiento (HAT). No se observó diferencias significativas entre las poblaciones de *P. hysterophorus* en la actividad de la enzima EPSPS con y sin diferentes dosis de glifosato. Las poblaciones R mostraron valores de actividad basal de la EPSPS entre 0.026 y 0.21 μmol μg^{-1} proteína TSP min^{-1} con respecto a las poblaciones S (0.024 y 0.025). Similar tendencia se observo en la actividad de la enzima EPSPS tratada con glifosato, donde un mayor I_{50} (μM glifosato) correspondió a mayores niveles de resistencia en poblaciones de *P. hysterophorus*. Una sustitución de aminoácido se encontró en la posición 106 en EPSPS, que consistió en un cambio de prolina a serina en Cu-R1, Do-R1 Do-R2. Los resultados obtenidos indican que los valores altos de resistencia observados estan determinados por el número de mecanismos de resistencia (TSR y NTSR) que poseen las diferentes poblaciones de *P. hysterophorus.*

INTRODUCTION

Herbicide resistance is an evolutionary phenomenon that allows resistant weed biotypes to be exposed to the normal dose of a herbicide undergoing any suffering growth alterations (Fernández *et al*., 2016). This biological phenomenon is favored by intensive herbicide applications with the same active ingredient or with the same mode of action (Neve *et al*., 2014; Evans *et al*., 2016). Glyphosate weed resistance is one of the world's most interesting cases, 35 glyphosate-resistant species have been detected and characterized (mainly using test dose response curves and shikimic acid accumulation) up to date (Heap, 2016).

Glyphosate ((N-phosphonomethyl)-glycine) is a post-emergent herbicide that is non-selective, highly systemic and widely used for weed control around the world (Franz *et al*.,1997; Székács and Darvas, 2012). It is well metabolized in plants and slow-acting with visible phytotoxic symptoms in sensitive plants at 10–20 days after application (Amrhein *et al.*, 1980; Shingh and Shaner, 1998; Monquero *et al*., 2004). It inhibits the shikimate pathway by inhibiting 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which catalyzes the synthesis reactions of aromatic amino acids involved in the formation of essential proteins in plants (Sammons and Gaines, 2014).

Glyphosate resistance selection is due to two different mechanisms known as non-target site resistance (NTSR) and target site resistance (TSR) (Shaner *et al.*, 2012; Sammons and Gaines, 2014). NTSR involves a reduced rate of herbicide in the meristem tissues due to limited absorption/translocation, and/or sequestration of the herbicide into compartments such as vacuoles (Michitte *et al.*, 2007; Ge et al., 2012; Vila-Aiub *et al.*, 2012). Metabolic pathways capable of degrading the herbicide to non-toxic compounds in plants also belong to these group mechanisms (De Prado and Franco, 2004; Cruz-Hipólito *et al.*, 2009,2011; Busi *et al.*, 2011; de Carvalho *et al.*, 2012; González-Torralva *et al.*, 2012; Alcántara-de la Cruz *et al*., 2016a). TSR has been produced by one or more mutations in the DNA sequence (González-Torralva *et al.,* 2014; Sammons and Gaines, 2014; Fernández *et al*., 2015; Yu *et al.*, 2015), or by the overexpression of the EPSPS protein by gene amplification (Gaines *et al.*, 2010; Salas e*t al*., 2012, 2015).

When growers reported noticing any deficiency in their weed control, they usually increased the glyphosate doses, which increased the pressure selection as well as

triggering the acquisition of a second resistance mechanism (Jasieniuk *et al.,* 1996; González-Torralva *et al*., 2012). Then, the level of weed resistance to glyphosate increased (Bostamam *et al.,* 2012).

Ragweed parthenium (*Parthenium hysterophorus* L.) is a troublesome annual weed of the *Asteraceae* family that is native to the Gulf of Mexico and other Latin American countries (Rosario *et al.,* 2013). Its prolific seed production (130,000–200,000 seeds m^{-2}), as well as the seeds's ability to persist in the soil and germinate over a wide range of temperatures, have contributed to the widespread distribution of ragweed parthenium in perennial and annual crops (orchards, citrus, soybean, corn) as well as in surrounding areas (Joshi, 1991; Pandey *et al*., 2003; Navie *et al*., 2004; Adkins and Shabbir, 2013). In addition, the subtropical environment of the Caribbean Islands (Cuba and Dominican Republic) allows year-round germination, growth, and reproduction of ragweed parthenium, which also contributes to its widespread distribution in the region. Glyphosate has been used repeatedly in perennial crop areas and fallow fields in the Caribbean Islands for many years to manage ragweed parthenium and other troublesome weeds. However, growers have recently observed reduced ragweed parthenium control with single or multiple glyphosate applications. Previous reports have documented glyphosate-resistant ragweed parthenium in Colombia (Rosario *et al*., 2013), Florida (southeast US) (Fernandez, 2013) and Dominican Republic (Jimenez *et al*.,2014), but in these three cases the causes of resistance to glyphosate have been inconclusive.

The main objective of this work is a survey of *P. hysterophorus* in Cuba and the Dominican Republic that had never been done before. The specific objectives were to determine (1) the level of glyphosate resistance of different accessions; (2) the possible NTSR and TSR mechanisms involved; and (3) to find out if the resistance genes may also increase the multiplicative or additive resistance levels in *P. hysterophorus*.

MATERIALS AND METHODS

Plant Material

In 2013, mature *P. hysterophorus* seeds were collected from plants not controlled with glyphosate at doses normally used (2 L ha^{-1}; 720 g ae ha^{-1}) in areas with perennial crops

in two Caribbean Islands. Seeds from Cu-S and Do-S accessions never exposed to glyphosate were collected from adjacent areas and used as a reference control (Table 1). Seeds collected from 25 mature plants were stored under laboratory conditions (25°C) for 2 weeks and then placed in paper bags at 4°C. Approximately 300 seeds of these accessions were sown directly into trays (40 × 60 × 15 cm), containing a mixture of sand and peat (2:1v/v) and placed in a greenhouse at 28/20°C day/night under a 16 h photoperiod with 850 µmol m^{-2} s^{-1} photon flux density, and 80% relative humidity. At the four leaf stage plants of all accessions were treated with glyphosate at 720 g ae ha^{-1} using a laboratory spray chamber equipped with a flat fan nozzle (TeeJet 8002 EVS) with a total output volume of 200 L ha^{-1} water at a pressure of 200 kPa. Four weeks after glyphosate treatment plant survival of the resistant accessions was estimated, and seed produced from surviving plants was collected and stored in paper bags for all subsequent trials. In the case of susceptible accessions (Cu-S and Do-S), no plant survival was observed 4 weeks after glyphosate treatment.

Table 1. History of different *P. hysterophorus* accessions used in this study.

Acssessions[a]	Location	Crop	Glyfosate (time of applications per year) number of applications per
Cu-R1	Ceiba	Orchards[c]	720 (2 or 3 times),>10
Cu-R2	Ceiba	Citrus[c]	720 (1 times),>10
Cu-R3	Arimao	Citrus	720 (2 times), unknown
Cu-S	Arimao	Road trails	No herbicide treatment
Do-R1	Villa Altagracia	Citrus[c]	900 (2 times),>15
Do-R2	San Cristobal	Citrus	900 (2 times),>15
Do-R3	Monseñor Nouel	Citrus	720 (2 times),>10
Do-R4	Maria T. Sanchez	Orchards	720 (1 times),>10
Do-S	Maria T. Sanchez	Road trails	No herbicide treatment

[a]Cu, *P.histerophorus* harvest in Cuba; Do, *P.histerophorus* harvest in Dominican Republic; [b]glyphosate glyphosate g ea ha^{-1}; [c] the last application was performed manually for every plant.

Dose-Response Assay

Seeds of putative resistant (Cu-R1, Cu-R2, Cu-R3, Do-R1, Do-R2, Do-R3, and Do-R4) and susceptible (Cu-S and Do-S) of the *P. hysterophorus* accessions were germinated in trays (12 × 12 × 6 cm) containing the same substrate as described before and placed in a

growth chamber of similar environmental conditions controlled as before. One week after germination, individual seedlings were transplanted into pots (6 × 6 × 8 cm) and grown under fluctuating 30/20°C day/night with a 14 h photoperiod and 850 μmol m^{-2} s^{-1} photon flux density, and 80% relative humidity. As glyphosate (EPSPS inhibitor) is used in early post-emergence, at the four leaf stage, resistant and susceptible *P. hysterophorus* seedlings were treated with increasing glyphosate doses: 0, 31.25, 62.5, 125, 250, 500, 1000, 2000, 4000, and 8000 g ae ha^{-1} (Roundup Energy 45% w/v, SL, Monsanto Spain). The experiment were conducted with 10 replications (one plant pot^{-1}) of each accession per herbicide dose, and the experiments were repeated twice. Thirty days after herbicide treatment, herbicide effects on plant survival (LD) and above-ground vegetative biomass (GR) were assessed.

Leaf Segment Shikimate Accumulation Assay

Leaf segments (50 mm diameter) were harvested from the youngest fully expanded leaf from a batch of 15 plants per *P. hysterophorus* accessions at the 4–6 leaf stage (Hanson *et al*., 2009). Approximately 50 mg of fresh tissue was transferred to 2 mL Eppendorf tubes containing 1 mL of 1 mM NH4H2PO4 (pH 4.4). Glyphosate was added to the tubes at the following concentrations: 0, 0.1, 0.5, 1, 5, 10, 50, 100, 200, 400, 500, 600, and 1000 μM. The Eppendorf tubes were incubated in a growth chamber during 24 h under the previously described conditions. After 24 h, the tubes were stored at −20°C until analysis. Eppendorf tubes were removed from the freezer and thawed at 60°C for 30 min. Two hundred and fifty micro liters of 1.25 N HCL was added to each tube, and placed at 60°C for 15 min. A 125 μL aliquot from each tube was pipetted into a new 2 mL Eppendorf tube, and 500 μL of periodic acid and sodium metaperiodate (0.25% [wt/v] each) was added. They were incubated at room temperature for 90 min, after which 500 μL of 0.6 N sodium hydroxide and 0.22 M sodium sulfite was added. The contents of all tubes were transferred to glass vials. Samples were measured in a spectrophotometer at 380 nm within 30 min. For each glyphosate concentration and accession, three replications were stablished and repeated twice.

^{14}C Glyphosate Absorption and Translocation

Absorption and translocation study was carried out following the methodology proposed by Cruz-Hipólito *et al*. (2011) The ^{14}C-glyphosate was mixed with commercially formulated glyphosate to prepare a solution with a specific activity of 0.834 kBq μL^{-1} and a glyphosate concentration of 1.8 g ae L^{-1} (360 g ae ha^{-1} in 200 L). *P. hysterophorus* plants at 4-leaf stage were treated with the radiolabeled herbicide by applying one droplet of 1 µL of glyphosate solution (0.834 kBq μL^{-1}) on the adaxial surface of the second leaf in each plant using a micropipette (LabMate). The ^{14}C-glyphosate unabsorbed in the treated leaf was removed with 3 mL of water: acetone solution (1:1, v/v) 96 h after droplet application. Preliminary assays with two accessions (Cu-R1 and Cu-S) studied had revealed that the glyphosate absorption leveled-off at 96 h after the droplet applications. The rinsate was mixed with 2 mL of scintillation liquid and analyzed by liquid scintillation spectrometry (LSS) (Scintillation Counter, Beckman LS 6500, Fullerton CA). The plants were separated into the treated leaf, rest of the shoot and root after being placed in cellulose cones. The plant tissue was dried at 60°C over 96 h and combusted in a biological sample oxidizer (Packard Tri Carb 307, Perkin-Elmer, Waltham, MA). The $^{14}CO_2$ evolved was trapped and counted in 18 mL of a mixture of Carbo-Sorb E and Permafluor (9:9, v/v) (Perkin-Elmer). Thus, over 95% of the total radioactivity applied was recovered. There were five replications and the experiment was arranged in a completely randomized design, and repeated twice. The proportion of absorbed herbicide was expressed as:

$$\textbf{\% absorbed} = \frac{\textbf{kBq in combusted tissue}}{\textbf{(kBq in combusted tissue + kBq in leaf washes))} \times \textbf{100}}$$

Glyphosate Metabolism

P. hysterophorus plants were treated with a glyphosate rate of 360 g ae ha^{-1} at 4–6 leaf stage. At 96 h after treatment (HAT), glyphosate and its metabolites, i.e., AMPA (aminomethylphosphonic acid), glyoxylate and sarcosine, were determined by reversed-polarity capillary electrophoresis following the methodology described by Rojano-Delgado *et al*. (2010). The calibration equations were established using non-treated plants and known concentrations of glyphosate and its metabolites, which were determined from their enclosed areas under the peaks in the electropherogram. The

average value for the amount of glyoxylate naturally produced by the plant was subtracted from the average of the produced or reduced amount after treatment of each accession (Rojano-Delgado *et al*., 2010). The experiment was arranged in a completely randomized design with four replications per accession and repeated three times.

EPSPS Enzyme Activity Assays

The enzyme extraction was conducted according to the protocol described by Dayan *et al.* (2015). Five gram of the leaf tissue of all *P. hysterophorus* accessions (Table 1) were ground to fine powder in a chilled mortar. Immediately after that, the powdered tissue was transferred to tubes containing 100 mL of cold extraction buffer (100 mM MOPS, 5 mM EDTA, 10% glycerol, 50 mM KCl and 0.5 mM benzamidine) containing 70 µL of β-mercaptoethanol and 1% in polyvinylpolypyrrolidone (PVPP). Samples were stirred and subsequently centrifuged for 40 min (18,000 g) at 4°C. The supernatant was decanted into a beaker using a cheesecloth. $(NH_4)_2SO_4$ was added to the solution to obtain 45% (w/v) concentration, with stirring during 30 min. After that, the mix was centrifuged at 20,000 g for 30 min at 4°C. The previous step was repeated to precipitate the protein in the extracts but in that case with a $(NH_4)_2SO_4$ concentration of 80% (w/v) stirring for 30 min. Finally, they were centrifuged at 20,000 g for 30 min at 4°C.

All the pellets were dissolved in 3 mL of extraction buffer and dialyzed in 2 L of dialysis buffer (30 mm, 1000-MWC dialysis tubing at 4°C on a stir plate) over 12 h. The protein concentrations were determined by Bradford assay (Bradford, 1976).

The assay for the determination of EPSPS activity followed the methodology described by Dayan *et al*. (2015) using the EnzCheck phosphate assay Kit (Invitrogen, Carlsbad, CA) to determine the inorganic phosphate release. The EPSPS activity from the nine accessions was determined in the presence and absence of glyphosate. The glyphosate concentrations used were: 0, 0.1, 1, 10, 100, and 1000 µM to determine the enzyme activity inhibition (I_{50}). The assay buffer was composed of 1 mM $MgCl_2$, 10% glycerol, and 100 mM MOPS, 2 mM sodiummolybdate and 200 mM NaF. The experiments were conducted with three replications of each accession per glyphosate concentration and repeated three times. EPSPS enzyme activity was expressed as percentage of enzyme activity in presence of glyphosate respect to the control (without glyphosate).

EPSP Synthase Gene Sequencing

For RNA extraction 100–200 mg of young leaves were taken from plants of each *P. hysterophor*us accession, and stored at −80°C for the extraction of RNA. Their tissue was ground in liquid nitrogen in a STAR-BEATER 412–0167 mill (VWR International Eurolab S.L., Barcelona, Spain). Total RNA was isolated from leaves as described by Pistón (2013), and the amount and quality were determined in a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Walthman, MA, USA). The synthesis to cDNA was from total RNA being adjusted to the same concentration in all the samples (50 ng μL^{-1}). An iScriptTM cDNA Synthesis Kit (Bio-Rad Laboratories, Inc. CA, USA) at 40 µL reaction volume was used following the manufacturer‘s instructions.

The PCR reactions were carried out with cDNA samples from each of the accession using the primers Bidens-F10 (5′- GGTTGTGGYGGTVTRTTTCC-3′) and Bidens-R11 (5′- GTCCCAASTATCACTRTGTTC-3′) based on EPSPS gene sequences described previously (Alcántara-de la Cruz *et al*., 2016b). PCR conditions were also as described (Alcántara-de la Cruz *et al*., 2016b). The PCR on cDNA amplified fragments of 462 bp in length, comprising the region of Thr-102 and Pro-106, which corresponds to the sequence of the EPSPS gene of Arabidopsis Klee *et al.* (1987), in which point mutations conferring resistance to glyphosate have been associated (Sammons and Gaines, 2014; Yu *et al.*, 2015).

The PCR fragments were cloned in the pGEM®-T Easy Vector System (Promega Biotech Ibérica, SL, Madrid, Spain) and transformed into competent cells of *E. coli* DH5α (Promega). Transformation was confirmed through PCR using the M13F and M13R primers as described (Alcántara-de la Cruz *et al*., 2016b). The colonies containing the length of the fragment were sequenced by the STABVIDA sequencing service (Caparica, Portugal). Five biological samples were used per accession providing 15 clones in all for each one. The quality and assembly of cDNA sequences and consensuses were determined employing the programs of SeqMan ProTM versión 11(DNASTAR; Wisconsin, USA) and Geneious® versión 8.1.8 (Biomatters Ltd, Auckland, New Zealand). The multiple sequences were aligned by means of the Muscle algorithm incorporated into SeqMan Pro versión 11.

Data Analysis

Dose-Response and EPSPS enzyme activity data were subjected to non-linear regression analysis (Seefeldt *et al*., 1995; Burgos *et al*., 2013) using a three-parameter log-logistic equation (Equation 1) to determine the glyphosate dose causing 50% reduction in above-ground vegetative biomass (GR_{50}), 50% mortality (LD_{50}), and inhibition of EPSPS activity by 50% (I_{50}).

$Y=\{[(d) / (1+(x/g)b)]\}$ (1)$Y=\{[(d) / (1+(x/g)b)]\}$ (1)

Where Y is the EPSPS activity, survival or above-ground biomass at herbicide x dose, d is the coefficient corresponding to the upper asymptote, b is the slope of the curve, and g is the herbicide rate at the point of inflection halfway (i.e., LD_{50}, GR_{50}, I_{50}).

Regression analyses were conducted using the drc package (Ritz et al., 2015) for the statistical environment R (R 3.2.4; R Core Team, 2015). Resistance indices were computed as R-to-S GR50 LD50, or I50 ratios. To test for a common GR_{50}, LD_{50}, or I_{50} for R and S accessions, i.e., Resistance Index equals to 1, a lack-of-fit test was used to compare the model consisting of curves with accessions-specific g values with a reduced model with common g (Ritz *et al.*, 2015).

Analysis of variance (ANOVA) was conducted using Statistix 9.0 (Analytical Software, USA) to test for differences between R and S accessions in shikimate accumulation at 1000 μM glyphosate in the leaf segment; and proportion of the different glyphosate metabolites; proportion of applied ^{14}C-glyphosate taken up by leaves, and proportions of absorbed ^{14}C-glyphosate remaining in the treated leaf, translocated to roots and to the rest of the plant at 96 HAT; and basal enzyme activity. Percentage data were previously transformed (arcsine of the square root) to meet model assumptions. Model assumptions of normal distribution of errors and homogeneous variance were graphically inspected. When needed, differences between means were separated using the Tukey HSD test.

RESULTS

Physiological Studies

Dose-response assays showed the existence of the first case of glyphosate-resistant weeds in the Caribbean (Cuba and Dominican Republic). The two susceptible weeds (Cu-S and Do-S) had similar susceptibility levels (Figures 1, 2; Table 2). The *P. hyterophorus* accessions from Cuba island had resistance index (RI) values (based on the GR_{50} and LD_{50} values) that ranged from 2.7 to 24.6, and 6.1 to 27.5 fold resistance, respectively, while on Dominican Republic island values were between 5.4 to 20, and 6.3 to 22.7 fold resistance, respectively (Table 2).

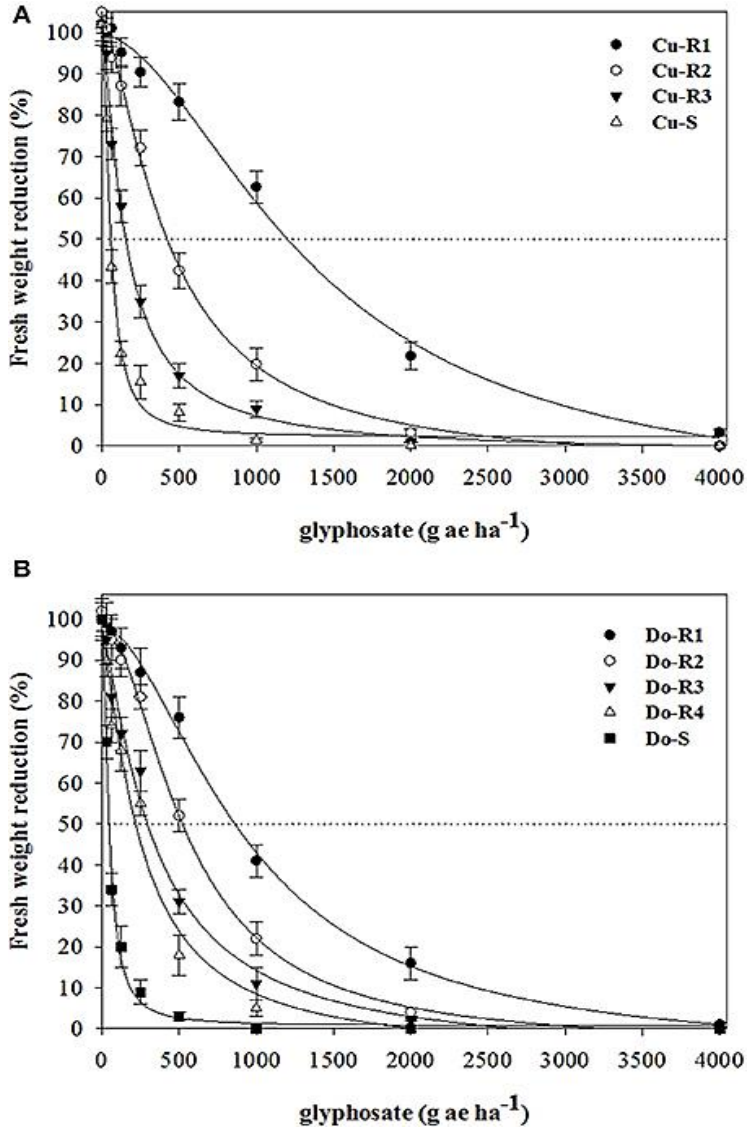

Figure 1. Shoot biomass in glyphosate-resistant and susceptible *P. hystherophorus* accessions from Cuba (A) and Dominican Republic (B) 30 days.after treatment. Symbols denoted mean (n = 10) ± standard errors of the mean.

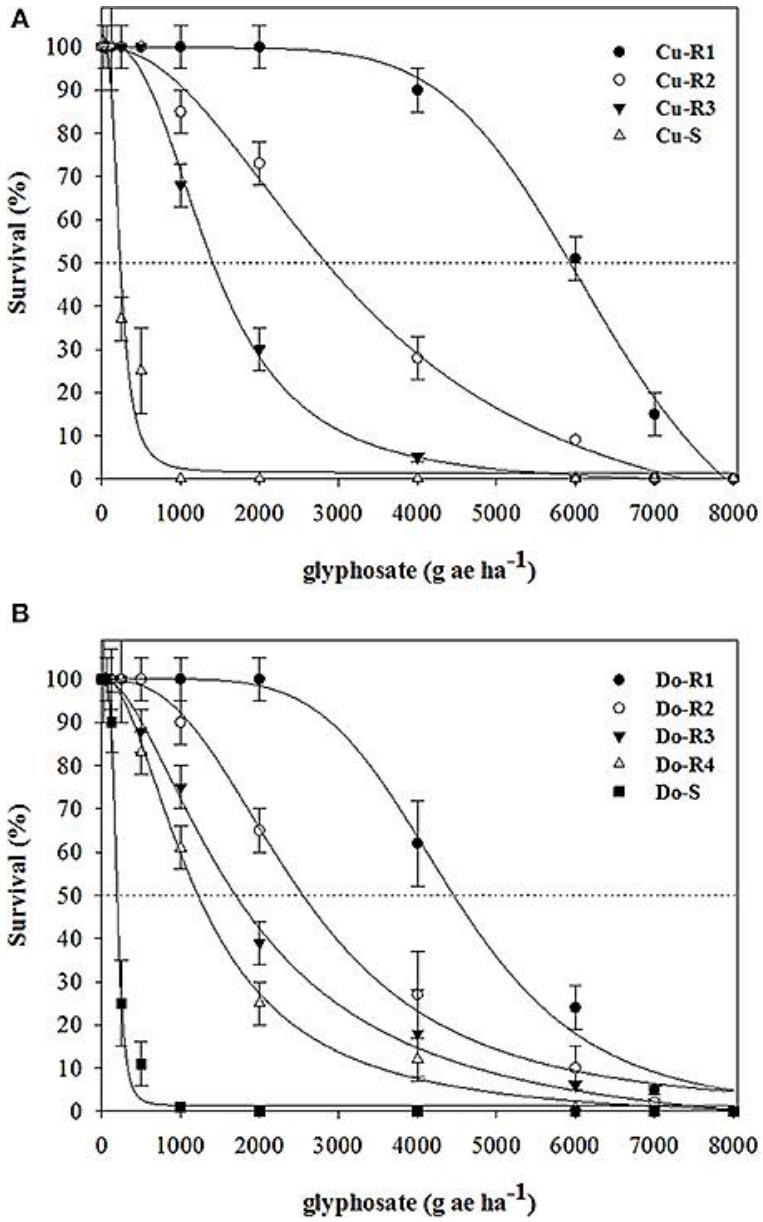

Figure 2. Survival plants in glyphosate-resistant and susceptible *P. hystherophorus* accessions from Cuba (A) and Dominican Republic (B) 30 days after treatment. Symbols denoted mean (n =10) ± standard errors of the mean.

Table 2. Parameters of the log-logistic equations used to calculate the glyphosate rates required for 50% survival (LD_{50}) and reduction fresh weight (GR_{50}) of the different accessions of *P. hyterophorus* from Cuba and Dominican Republic.

Accessions	Survival[a] (%)						Fresh weight reduction[b] (%)					
	d	*b*	R^2	LD_{50} (g ae ha^{-1})	RI	*p*	*d*	*b*	R^2	GR_{50} (g ae ha^{-1})	RI	*p*
Cu-R1	99.8	6.1	0.98	6364 ± 122	27.5	<0.0001	99.4	1.8	0.99	1370 ± 191	24.5	<0.0001
Cu-R2	98.9	2.9	0.99	2794 ± 90	12.0	<0.0001	103.0	1.5	0.95	437 ± 28	7.8	<0.0001
Cu-R3	100.9	2.6	0.99	1415 ± 55	6.1	<0.0001	103.3	1.3	0.96	151 ± 13	2.7	0.003
Cu-S	102.7	3.1	0.97	232 ± 23	–	–	103.2	1.7	0.98	56 ± 6	–	–
Do-R1	100.1	5.1	0.96	4456 ± 76	22.7	<0.0001	98.2	1.8	0.98	939 ± 25	20.0	<0.0001
Do-R2	99.9	2.7	0.98	2550 ± 92	13.0	<0.0001	99.6	1.8	0.99	547 ± 30	11.6	<0.0001
Do-R3	100.7	1.7	0.99	1821 ± 63	9.3	<0.0001	97.9	1.3	0.99	339 ± 27	7.2	<0.0001
Do-R4	100.9	1.9	0.99	1242 ± 65	6.3	<0.0001	96.4	1.3	0.96	255 ± 33	5.4	<0.0001
Do-S	100.5	4.5	0.97	196 ± 8	–	–	100.6	1.7	0.98	47 ± 4	–	–

[a]*For* $Y = \{(d) / [1 + (x/ LD_{50}) \exp b]\}$ *Where Y is the survival expressed as a percentage of the untreated control, d is the coefficient corresponding to the upper asymptote, b is the slope of the curve in* LD_{50}, LD_{50} *is the herbicide rate at the point of inflection halfway, and x is the herbicide dose.*
[b]*For* $Y = (d) / [1 + (x/ GR_{50}) \exp b]$ *Where Y is the above-ground weight expressed as a percentage of the untreated control, d is the coefficient corresponding to the upper asymptote, b is the slope of the curve in* GR_{50}, GR_{50} *is the herbicide rate at the point of inflection halfway, and x is the herbicide dose.*

The fact that plants treated with glyphosate increase shikimic acid accumulation in leaf disks due to the inhibition of EPSPS activity led us to carry out the experiment depicted in Figures 3A, B. Considering the values obtained *in vivo* (GR_{50} and LD_{50}) and the shikimic acid accumulation in leaf disks at 1000 µM of glyphosate, the resistance order of the *P. hystherophorus* accessions was Cu-R1 ≥ Do-R1 > Do-R2 > Cu-R2 > Do-R3 > Do-R4 > Cu-R3 >> Cu-S ≥ Do-S. There were significant differences at 1000 µM glyphosate between R and S accessions of Cuba (p =0.0013, DF =3, n =12) and Dominican Republic (p = 0.0008, DF = 4, n =15).

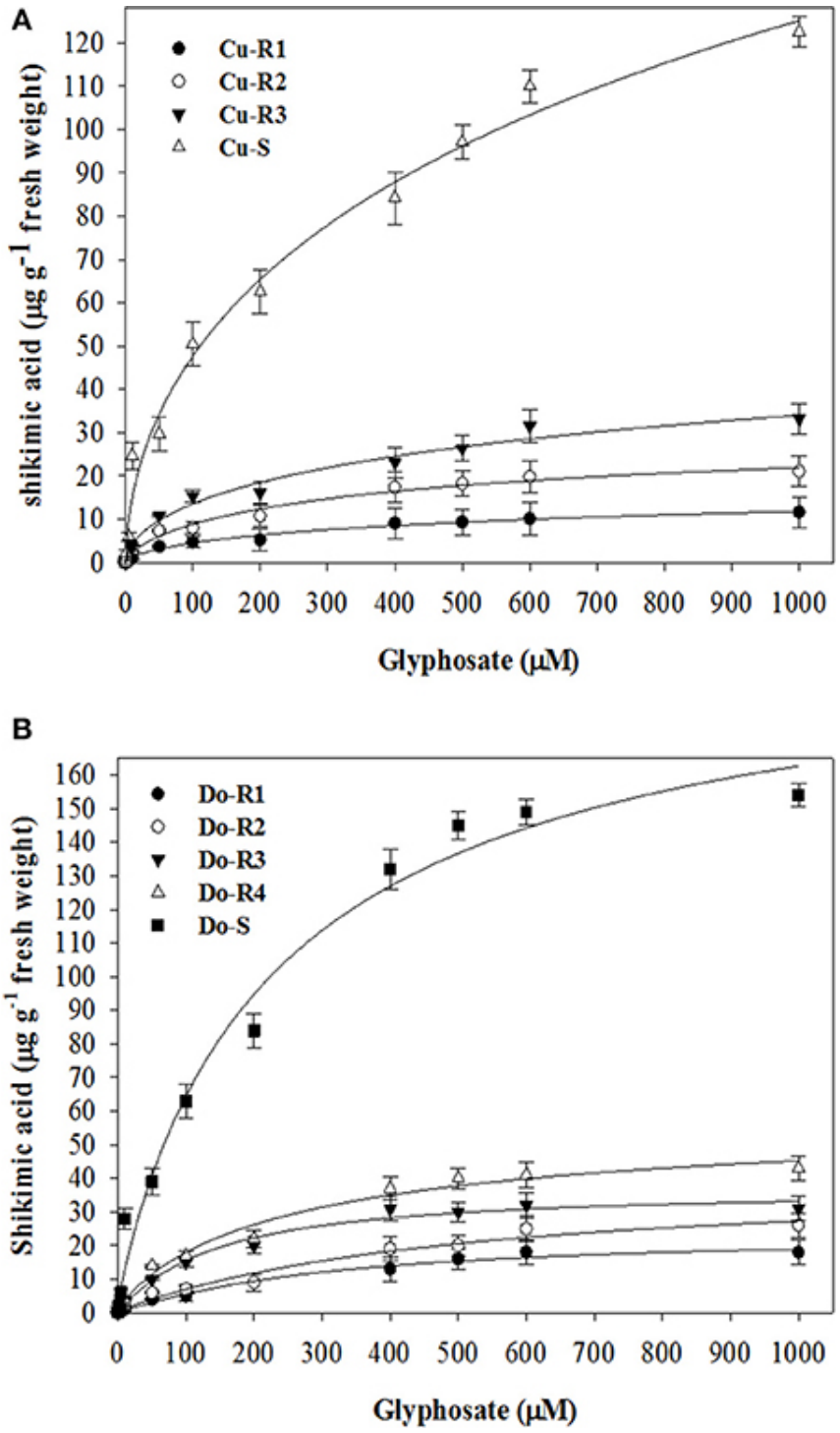

Figure 3. Shikimic acid accumulation plants from Cuba (A) and Dominican Republic (B) accessions of *P. hysterophorus*. Symbols denoted mean (n =3) ± standard errors of the mean.

There were marked differences in glyphosate absorption between the resistant and susceptible glyphosate *P. hysterophorus* accessions at 96 h after treatment (HAT) (p = 0.0001, DF =8, n =45) (Table 3). All accessions obtain maximum absorption at 96 HAT, and the two susceptible accessions absorbed an average of 80.5%, while the

resistance accessions absorbed an average of 59.2% of ^{14}C-glyphosate which was recovered.

Table 3. ^{14}C-glyphosate absorption (% of recovered radioactivity) and translocation (% of absorbed radioactivity) in the different *P.hysterophorus* accessions at 96 h after treatment (HAT).

Accessions	Absorption[a] ($p = 0.0001, DF = 8, n = 45$)	Translocation		
		Treated leaf ($p = 0.0003, DF = 8, n = 45$)	Rest of shoot ($p = 0.0001, DF = 8, n = 45$)	Root ($p = 0.0004, DF = 8, n = 45$)
Cu-R1	59.3 ± 4.9 BC	77.9 ± 5.7 AB	12.1 ± 2.1 BCD	10.0 ± 2.3 BC
Cu-R2	60.2 ± 2.1 BC	82.4 ± 4.2 A	9.3 ± 1.9 D	8.3 ± 3.4 BCD
Cu-R3	56.8 ± 3.9 C	80.1 ± 3.9 AB	15.7 ± 3.4 B	4.2 ± 1.2 D
Cu-S	82.2 ± 6.7 A	35.5 ± 2.3 C	41.6 ± 6.2 A	22.9 ± 4.8 A
Do-R1	63.1 ± 6.8 B	78.3 ± 6.7 AB	10.5 ± 2.7 CD	11.2 ± 2.1 B
Do-R2	55.9 ± 7.8 C	79.3 ± 3.4 AB	16.2 ± 4.9 B	4.5 ± 1.4 D
Do-R3	60.4 ± 3.7 BC	75.6 ± 5.1 B	14.1 ± 3.8 BC	10.3 ± 3.8 B
Do-R4	58.4 ± 2.3 BC	81.4 ± 6.3 A	12.7 ± 4.3 BCD	5.9 ± 2.7 CD
Do-S	78.8 ± 5.6 A	39.1 ± 1.9 C	37.8 ± 2.3 A	23.1 ± 5.6 A

[a]*Over 95% of the total radioactivity applied was recovered.*
Mean value (n = 5) ± standard error. Means on a same column followed by the same letter were not significantly different at α = 0.05.

Translocation assays suggest marked differences at 96 HAT between the Cu-S and Do-S accessions compared to the Cu-R1, Cu-R2, Cu-R3, Do-R1, Do-R2, Do-R3, and Do-R4 ones in treated leaf ($p = 0.0003, DF = 8, n = 45$), rest of the shoots ($p = 0.0001$, $DF = 8$, $n = 45$), and root ($p = 0.0004$, $DF = 8$, $n = 45$) (Table 3). There were no significant differences in translocation between the two susceptible accessions (Cu-S and Do-S) from Caribbean Islands. But there were small significant differences in the resistant accessions (Cu-R1, Cu-R2, Cu-R3, Do-R1, Do-R2, Do-R3, and Do-R4). Nonetheless, the high amount of ^{14}C-glyphosate in each resistant accession remained in the treated leaf. Due to differences in levels of glyphosate resistance between the *P. hysterophous* resistant accessions, we suspect that other mechanisms could be involved (Tables 2, 3, Figure 3).

Biochemical Studies

Previous tests demonstrated that the highest glyphosate translocation and metabolism was reached at 96 HAT in the *P. hysterophorus* accessions (unpublished data). There were significant differences at 96 HAT in glyphosate metabolism levels between accessions ($p = 0.0014, DF = 8, n = 36$). Glyphosate levels decreased, whereas glyphosate metabolites (AMPA, glyoxylate and sarcosine) increased at 96 HAT in the Cu-R1, Do-R1, Do-R2, Cu-R2, and Do-R3 accessions. Higher glyphosate levels

remained in the Cu-R3 and Do-R4 (low resistance), and very high one in the Cu-S and Do-S (susceptible) accessions. In these last accessions, sarcosine was not detected (Table 4). These results can also explain the low level of resistance of the accession (Cu-R3 and Do-R4) with a single resistance mechanism, while the other glyphosate resistant accessions have at least two mechanisms (Tables 3, 4).

Table 4. Glyphosate metabolism expressed as a percentage of total glyphosate and its metabolites in *P. hystherophorus* susceptible and resistant-glyphosate accessions at 96 HAT.

Accessions	Glyphosate ($p = 0.0014, DF = 8, n = 36$)	Metabolites		
		AMPA ($p = 0.0003, DF = 8, n = 36$)	Glyoxylate ($p = 0.0001, DF = 8, n = 36$)	Sarcosine ($p = 0.0002, DF = 8, n = 36$)
Cu-R1	9.80 ± 1.70 D	60.54 ± 1.32 B	18.14 ± 0.32 C	11.52 ± 0.96 A
Cu-R2	21.12 ± 0.93 C	55.31 ± 1.57 B	20.80 ± 0.51 AB	2.77 ± 0.31 E
Cu-R3	73.42 ± 3.63 B	26.14 ± 0.26 C	0.44 ± 0.02 E	ND
Cu-S	91.82 ± 4.81 A	7.68 ± 0.33 E	0.50 ± 0.02 E	ND
Do-R1	11.83 ± 0.74 D	58.94 ± 2.79 B	21.74 ± 0.97 A	7.49 ± 0.27 C
Do-R2	11.37 ± 0.80 D	64.70 ± 2.93 A	18.54 ± 0.83 C	5.39 ± 0.15 D
Do-R3	9.56 ± 0.72 D	60.95 ± 2.71 B	20.36 ± 0.94 B	9.13 ± 0.53 B
Do-R4	71.21 ± 1.06 B	20.05 ± 2.20 D	7.28 ± 0.93 D	1.01 ± 0.71 F
Do-S	90.68 ± 4.39 A	8.86 ± 1.06 E	0.46 ± 0.03 E	ND

Mean value (n = 4) ± standard error. Means on a same column followed by the same letter were not significantly different at α = 0.05.
ND, non-detected; AMPA, aminomethylphosphonic acid.

The EPSPS enzymes of all the accession plants were inhibited by glyphosate. The I_{50} (herbicide dose which reduces the enzyme activity to 50%) values were different in all accessions, ranging between approximately 47.65 in Cu-R1, 25.2 in Do-R1, 22.1 in Do-R2, 1.4 in Cu-R2, 1.2 in Do-R3, 1.2 in the Cu-R3, and 1.1-fold resistance in Do-R4 accessions relative to their susceptible accession, respectively (Figure 4, Table 5). These results were in accordance with the *in vivo* resistance level shown for the different accessions, and suggest that multiple mechanisms in the target-site could be expressed in these accessions.

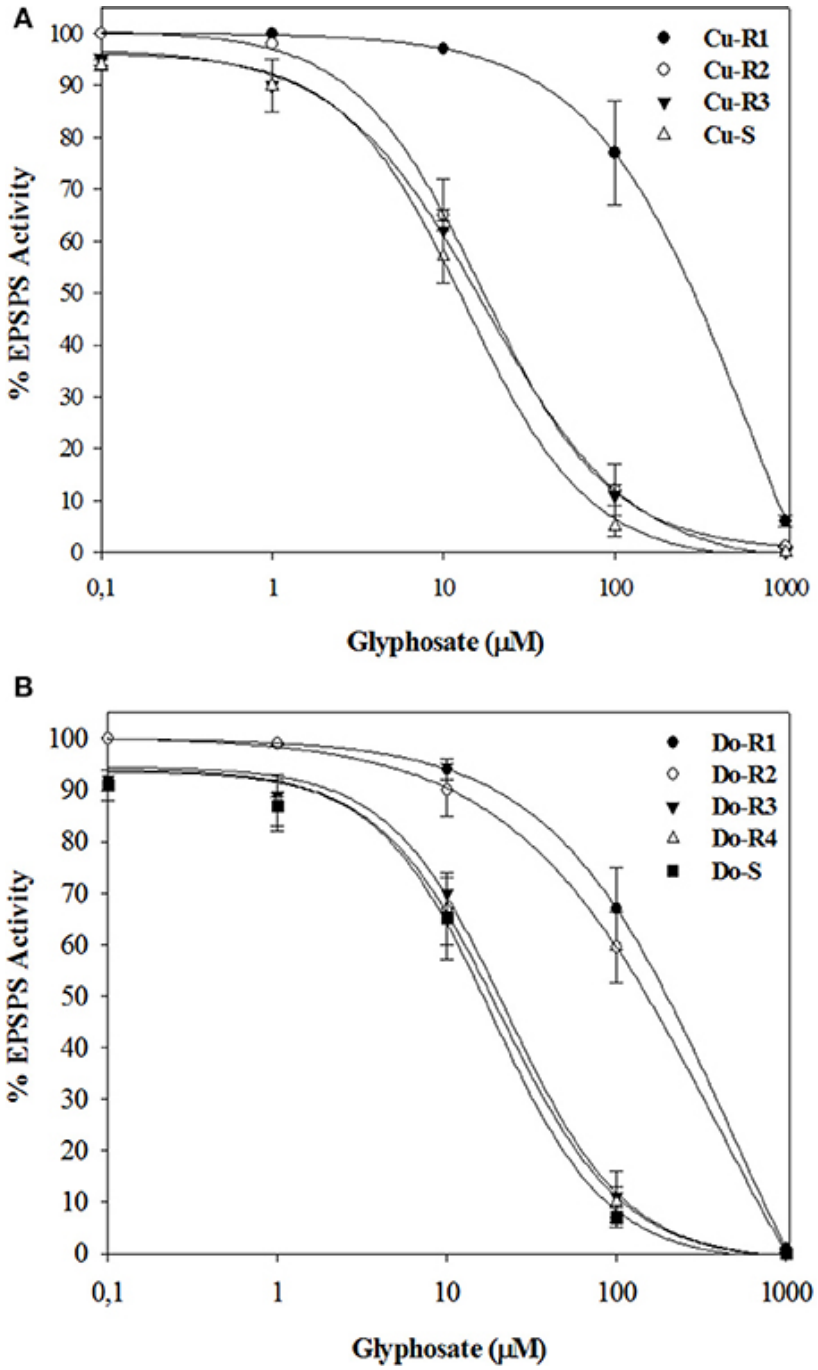

Figure 4. EPSPS enzyme activity expressed as percentage of the untreated control in leaf extracts of plants from Cuba (A) and Dominican Republic (B) accessions of *P. hysterophorus*. Symbols denoted mean (n = 3) ± standard errors of the mean.

Table 5. Parameter estimates of the equation used to calculate the sensitivity of EPSPS enzyme activity to glyphosate in extracts from leaf tissue of the different accessions of *P. hyterophorus* from Cuba and Dominican Republic.

Accessions	*d*	*b*	R^2	I_{50} (μM)[a]	RI	*P*
Cu-R1	100.1	0.9	0.97	646.2 ± 35.8	47.6	<0.0001
Cu-R2	99.8	0.8	0.96	18.9 ± 1.4	1.4	0.1902
Cu-R3	97.0	1.0	0.99	17.4 ± 2.8	1.2	0.2186
Cu-S	96.2	1.2	0.98	13.6 ± 2.2	–	–
Do-R1	100.0	0.8	0.99	468.1 ± 22.0	25.2	<0.0001
Do-R2	100.4	0.7	0.99	410.7 ± 26.1	22.1	<0.0001
Do-R3	94.5	1.2	0.98	22.6 ± 1.5	1.2	0.3714
Do-R4	94.0	1.2	0.96	20.8 ± 6.1	1.1	0.6042
Do-S	93.6	1.2	0.99	18.5 ± 5.7	–	–

[a]*For* $Y = \{(d) / [1 + (x/ I_{50}) \exp b]\}$ *Where Y is the EPSPS activity, d is the coefficient corresponding to the upper asymptote, b is the slope of the curve in* I_{50}, I_{50} *is the herbicide rate at the point of inflection halfway, and x is the herbicide dose.*

The basal activity of EPSPS enzyme (without glyphosate) in the resistant accessions was between 0.026 and 0.21 μmol μg^{-1} protein min^{-1}, while the susceptible accessions (Cu-S and Do-S) were lower with 0.024 and 0.025 μmol μg^{-1} protein min^{-1}, respectively (Figure 5). There were market differences between accessions in both Cuba (p = 0.0001, DF = 3, n = 12), and Dominican Republic (p = 0.0002, DF = 4, n = 15). The Cu-R1, Do-R1, and Do-R2 exhibited 8.8, 7.2, and 4.8-times higher basal enzyme activities than their susceptible accessions, respectively. For Cu-R2, Do-R3, Do-R4, and Cu-R3 accessions the values were similar to those found for their susceptible accessions, respectively.

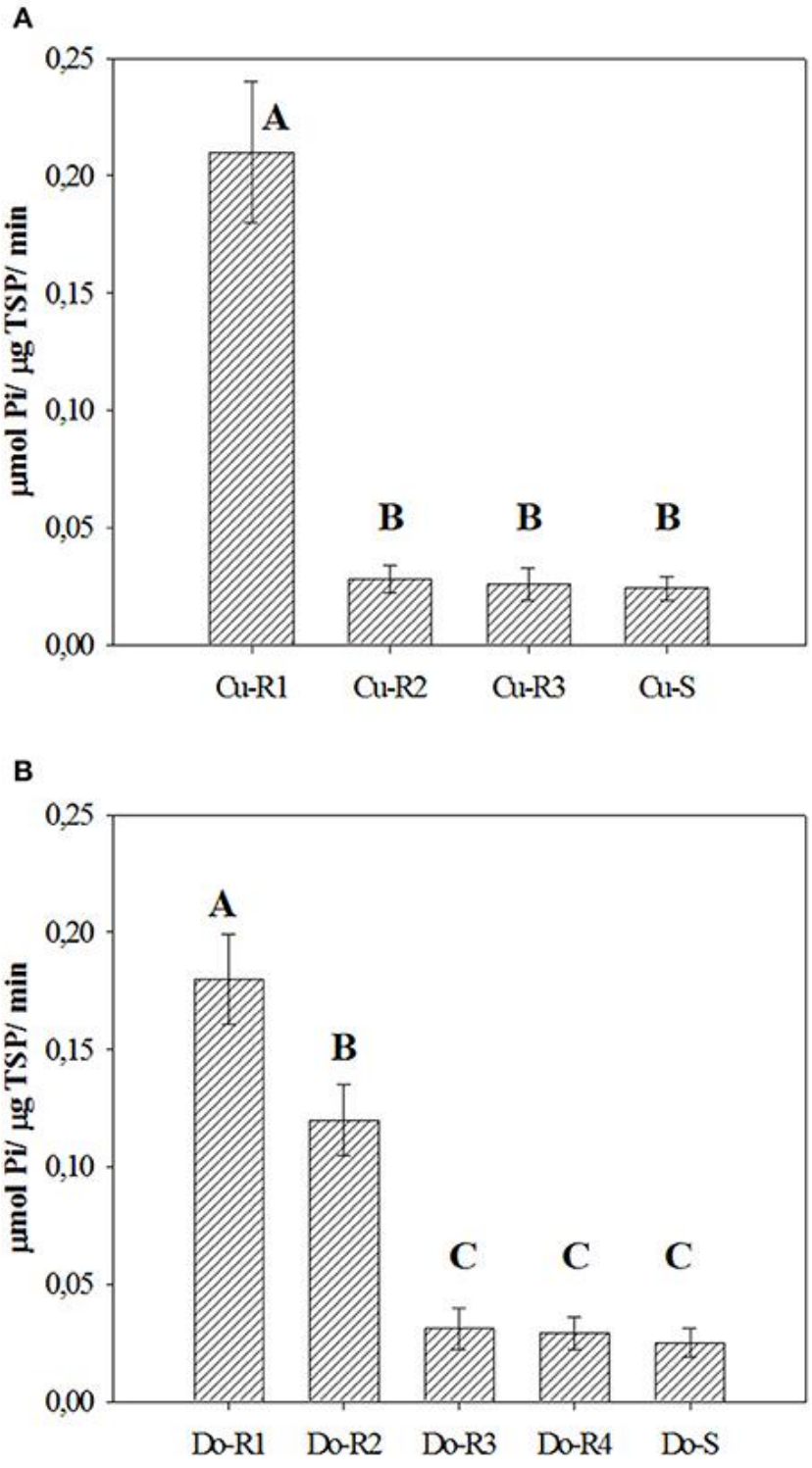

Figure 5. Basal EPSPS activity for glyphosate-susceptible and resistant from Cuba (A)and Dominican Republic (B) accessions of *P. hysterophorus*. Vertical bars are ± standard errors of the mean. Means by the same letter were not significantly different at $\alpha = 0.05$

Molecular Studies

A total of 462 bp of the EPSPS gene of *P. hysterophorus* plants of resistant and susceptible accessions were sequenced. The fragments were aligned and numbered based on a published EPSPS sequence of *Arabidopsis thaliana* (L.) Heynh. (GenBank: CAA29828.1). The resistant accessions of *P. hysterophorus* Cu-R1 from Cuba, and Do-R1 and Do-R2 from Dominican Republic, showed an amino acid substitution at position 106 consisting of a Proline to Serine (Figure 6).

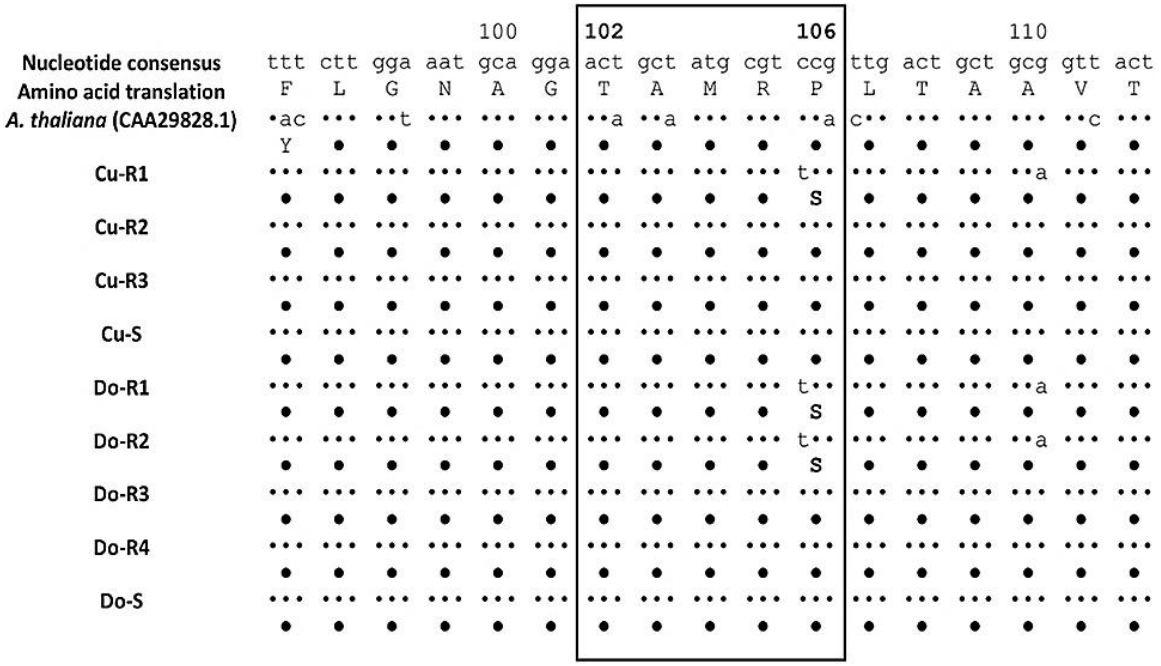

Figure 6. Partial protein sequence alignment of the EPSPS gene of resistant and susceptible *P. hysterophorus* plants.

The box comprising the region of Thr-102 and Pro-106 point mutations associated to confer glyphosate resistance. The points indicate homology between the different sequences.

DISCUSSION

P. hysterophorus is universally recognized for its widespread distribution and high seed production, commonly known as the parthenium weed. Parker (1989) identified two biotypes with different flowering patterns in Mexico (Caribbean area), and they were genetically distinct biotypes (Clermont and Toogoolawah). Moreover, Hanif *et al*.

(2011) found that these two biotypes differed in their morphology and reproductive behavior; in particular, the Toogoolawah biotype shows a greater tendency toward self-pollination, but these biotypes can also present out-crossing. It makes sense that it would reproduce prolifically and that higher resistance levels due to accumulation of multiple mechanisms, by multiple crossings, would proliferate within populations (Table 6).

Table 6. Summary of glyphosate resistance mechanisms accumulated by *P. hysterophorus* accessions studied in this work.

Accessions	GR_{50}[a]	LD_{50}[a]	Absorption and translocation	Glyphosate metabolism	Enhanced EPSPS basal activity[b]	EPSPS (I_{50}[b])	Pro106Ser
Cu-R1	1370	6364	Low	High	Yes	High	Yes
Cu-R2	437	2794	Low	High	No	Low	No
Cu-R3	151	1415	Low	Medium	No	Low	No
Cu-S	56	232	High	Low	No	Low	No
Do-R1	939	4456	Low	High	Yes	High	Yes
Do-R2	547	2550	Low	High	Yes	High	Yes
Do-R3	339	1821	Low	High	No	Low	No
Do-R4	255	1242	Low	Medium	No	Low	No
Do-S	47	196	High	Low	No	Low	No

[a]*glyphosate g ae ha*$^{-1}$; [b]*glyphosate* μM.

Glyphosate has been used repeatedly in perennial crop areas and fallow fields in the Caribbean Islands for many years to manage *P. hysterophorus* and other troublesome weeds. However, using glyphosate alone without any additional alternative and/or IWM (Integrated Weed Management) led to the emergence of glyphosate-resistant weeds early in the second decade of the 21st century (Tables 1, 2). Herbicide response between different locations depends on local ecological factors, such as a variation in soil type, tillage practices, types of crops, fertilizers, etc., (Shaner and Beckie, 2014; Jussaume and Ervin, 2016). Our results showed different glyphosate resistance levels between the *P. hysterophorus* accessions. This differences could be addressed to the use of different glyphosate formulations and dose rate, the application technique (manual or mechanical) employed by farmers, and the agro environment conditions (Neve *et al.*, 2014; Renton *et al.*, 2014; Jussaume and Ervin, 2016; Matzrafi *et al.*, 2016; Owen, 2016). It has been shown that an increase in the relative humidity and temperature increases the glyphosate absorption, translocation, and toxicity in many weed species (Ge *et al.*, 2011; Hatterman-Valenti *et al.*, 2011; Vila-Aiub *et al.*, 2012; Santos *et al.,* 2016). This research also revealed that the low GR_{50} and LD_{50}values for the susceptible accessions showed that glyphosate has been a very effective tool for farmer for over 15

years, as has been shown in *P. hysterophorus* from Colombia, Dominican Republic, and Florida (Fernandez, 2013; Rosario *et al*., 2013; Jimenez *et al*., 2014).

Plants with low levels of GR_{50} and LD_{50} are related to an increased inhibition of EPSPS activity and a greater accumulation of shikimic acid (Shaner *et al*., 2005; Gaines *et al.,* 2010; Fernández *et al.,* 2015). High levels of resistance (RI) and low shikimic acid accumulation observed in the different *P. hystherophorus* accessions were consistent with those of plants which have acquired resistance to the addition of more than one NTSR and/or TSR mechanisms, as has been shown in dicotyledonous weed species such as *Amaranthus tuberculatus* (Nandula *et al.*, 2013), *Conyza sumatrensis* (González-Torralva *et al.*, 2014), and several grass weed species (Michitte *et al.*, 2007; de Carvalho *et al*., 2012; Fernández *et al*., 2015).

According to Shepherd and Griffiths (2006), a cuticular wax layer provides a protective barrier for a wide range of abiotic stresses (pesticide). Resistant and tolerant plants have displayed a cuticle containing a massive amount of epicuticular wax which forms a nonuniform 3D cover as has been revealed by scanning electron micrographs (De Prado *et al.,* 2005; Wang and Liu, 2007; Rojano-Delgado *et al*., 2012; *Alcántara-de la Cruz et al*.,2016a).The limited glyphosate absorption by the resistant *P. hysterophorus* accessions was likely to have been due to differences in outer leaf surfaces. Different translocation can be explained by ^{14}C-glyphosate and/or its metabolite accumulation in the tips of the resistant treated leaves, while ^{14}C was removed from the susceptible treated leaves (Table 3). Since the first case of glyphosate resistance was detected in a population of *Lolium rigidum* in Australia (Powles *et al*., 1998), both previously mentioned mechanisms were considered responsible for this resistance (Wakelin *et al*., 2004; Michitte *et al.*, 2007; Preston and Wakelin, 2008; de Carvalho e*t al.*, 2012; González-Torralva *et al.*, 2012, 2014; Nandula *et al.,* 2013; Fernández *et al.*, 2015). Subsequent studies in the main dicot and monocotyledonous glyphosate-resistant weeds seem to have demonstrated that the main NTSR mechanism involved in their resistance is due to a lesser glyphosate absorption and/or -translocation (Feng *et al.*, 2004; Michitte *et al*., 2007; de Carvalho *et al.,* 2012; González-Torralva *et al*., 2012, 2014; Vila-Aiub *et al*., 2012; Nandula *et al.*, 2013; Adu-Yeboah *et al.*, 2014).

In some plants, the glyphosate degradation to glyoxylate and AMPA is carried out by a glyphosate oxidoreductase (GOX) and the glyphosate degradation to sarcosine and inorganic phosphate by a C–P lyase. These steps have been reported by some authors such as Liu *et al.* (1991); Komoba *et al.* (1992); Saroha *et al*. (1998); Al-Rajab and Schiavon (2010), and Duke (2012) among others. However, only a few works unify these two degradation pathways to explain the glyphosate metabolism in leguminous plants and weeds (de Carvalho *et al*., 2012; Rojano-Delgado *et al*., 2012). Some authors consider that metabolism has a low contribution to the resistance or, even more, that it is nonexistent (Saroha et al., 1998; Feng et al., 2004; Duke, 2012; Sammons and Gaines, 2014). However, the fact is that this mechanism involves a decrease in the concentration of the herbicide glyphosate around the target-site, diminishing the EPSPS inhibition rate (Duke, 2012; Sammons and Gaines, 2014; Alcántara-de la Cruz *et al*., 2016a). The GOX gene that encodes the glyphosate metabolizing enzyme glyphosate oxidoreductase was cloned from *Achromobacter* sp. *strain* LBAA (Barry *et al*.,1994). Neither plant GOX nor the gene(s) encoding it have been isolated or elucidated. A plant gene encoding GOX might be useful in genetically engineering crops and weed resistance development (Duke, 2012; Rojano-Delgado *et al.*, 2012). Some researchers have proposed additive effects of concurrent glyphosate resistance mechanisms in the same weed species (Gaines *et al*., 2010; Yu *et al*., 2010; Bostamam *et al*., 2012; Rojano-Delgado *et al.*, 2012), which would explain the difference in the resistance between accessions keeping the same percentage of metabolic degradation (Table 6). However, genetic basic controlling absorption/translocation and/or metabolism including genes involved have not been identified so far (Yuan *et al*., 2006; Delye, 2013; Délye *et al.*, 2013). This could be a highly promising research area in the future.

Taking into account these results, resistance could be associated with target enzyme overexpression. Some species as ryegrass (Yu *et al*., 2007; Dayan *et al*., 2012) have shown differences in the basal EPSPS enzyme activity as a consequence of the EPSPS gene overexpression. However, in the *L. perenne* spp. *multiflorum* population from Arkansas, no differences were observed in the I_{50} values, which could be explained as a lack of effective mutations in the binding site of the enzyme (Salas *et al*., 2015). In our case, some accessions are candidates to possessing an effective mutation (Figure 6, Table 6) or a possible EPSPS overexpression, explaining their high resistance to

glyphosate compared to other accessions. We are aware of that fact, and effective research is currently in progress to characterize the EPSPS overexpression resistance mechanism involving these accessions.

Results reported here are in agreement with previous works, in which the Proline to Serine substitution was found to confer glyphosate resistance in other weed species such as *A. tuberculatus, C. sumatrensis, Echinochloa colona*; *L. perenne* spp. *multiflorum* and *L. rigidum* (Bostamam *et al*., 2012; *González-Torralva et al.*, 2012, 2014; Nandula *et al*., 2013; Fernández *et al*., 2015; Han *et al.*, 2016). However, mutations in the Pro-106 position generally provide only a low level (2–4-fold) of glyphosate resistance (Kaundun *et al.*,2011). Here, *P. hysterophorus* accessions that presented Pro-106 mutation had a resistance factor of >12. These three accessions (Cu-R1, Do-R1, and Do-R2) were more highly resistant to glyphosate as a result of showing different concurrent resistance mechanisms, including reduced absorption and translocation, glyphosate metabolism, and EPSPS gene mutation.

In some species, at least more than one glyphosate resistance mechanism have been reported, such as *A. tuberculatus* (Nandula *et al.*, 2013), *L. rigidum* (Bostamam *et al*., 2012), *L. perenne* spp. *multiflorum* (González-Torralva *et al*., 2012), and *L. perenne* (Ghanizadeh *et al*., 2015) populations which exhibited a mutation in Pro-106 position, and a reduced translocation. Besides, other species such as *Digitaria insularis* presented a pool of mechanisms (absorption, translocation, metabolism, and EPSPS gene mutation) (de Carvalho *et al.*, 2012). The involvement of several resistance mechanisms is evident when looking at the resistance levels of accessions Cu-R2, Cu-R3, Cu-R4, Do-R3, Do-R4, and Do-R5 of *P. hysterophorus*, which did not show any mutation in the Pro-106 position. This is the first time that a mutation in the target-site has been reported in glyphosate-resistant *P. hysterophorus*.

In summary, we have confirmed resistance to glyphosate in different *P. hysterophorus* accessions harvested in the Caribbean Islands. Their resistance levels depend on the different resistance mechanisms (NTSR and TSR) that are accumulated by these accessions (Table 6), due to increasing selection pressure and out-crossing. The evolution of multiple mechanisms found in this resistance species is worrying. The farmers should implement manage practices such as the use of cover crops, which prevent soil erosion and allow the use of grazing, as well as the use of other non-

selective herbicides in an integrated weed management (IWM) to facilitate the reduction and suppression of herbicide-resistant accessions.

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CAPÍTULO III

Identifying *Chloris* Species from Cuban Citrus Orchards and Determining Their Glyphosate-Resistance Status

Bracamonte, E. R., Fernández-Moreno, P. T., Bastida, F., Osuna, M. D., Alcántara-de la Cruz, R., Cruz-Hipolito, H. E., & De Prado, R. (2017). Identifying Chloris Species from Cuban Citrus Orchards and Determining Their Glyphosate-Resistance Status. *Frontiers in plant science*, *8*, 1977.

ABSTRACT

The *Chloris* genus (Poaceae: *Chloridoidae*) is a C_4 photosynthetic species mainly distributed in tropical and subtropical regions. Populations of three *Chloris* species occurring in citrus orchards in central Cuba under long-lasting glyphosate-based weed management, were studied for taxonomic identification and resistance to glyphosate status and mechanisms. Morphological and molecular analyses allowed these species to be identified as *C.ciliata* Sw*., C.elata* Desv. and *C.barbata* Sw. Based on the glyphosate rate causing 50% mortality (LD_{50}) of treated plants, glyphosate resistance (R) was confirmed only in *C. elata*, The R population was 6.14-fold more resistant compared to the susceptible (S) population. In addition, R plants of *C. elata* accumulated 4.6 fold less shikimate after glyphosate application than S plants. Meanwhile, populations of *C. barbata* and *C.ciliata* with or without glyphosate application history showed similar LD_{50} values and shikimic acid accumulation rates, demonstrated that these species present tolerance to glyphosate. Plants of R and S populations of *C. elata* differed in ^{14}C-glyphosate absorption and translocation. The R population exhibited 27.3-fold greater 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS) activity than S population, due to a target site mutation corresponding to Pro-106-Ser substitution fund in the EPSPS gene. These reports evidence the innate tolerance to glyphosate of *C. barbata* and *C. ciliata,* and confirm the resistance of *C. elata* to this herbicide, showing that both non-target site and target-site mechanisms are involved in its resistant behavior. This is the first case of herbicide resistance in Cuba.

RESUMEN

El género *Chloris* (Poaceae: *Chloridoidae*) es una especie fotosintética C_4 distribuida principalmente en regiones tropicales y subtropicales. Las poblaciones de tres especies de *Chloris* que se encuentran en huertos de cítricos del centro de Cuba, bajo una larga historia de manejo de malezas a base de glifosato, fueron estudiadas por identificación taxonómica y su resistencia al glifosato mediante la caracterización de sus mecanismos de resistencia. Los análisis morfológicos y moleculares permitieron identificar a estas especies como *C. ciliata* Sw., *C. elata* Desv. y *C. barbata* Sw. Con base en la tasa de glifosato que causa el 50% de mortalidad de las plantas tratadas, la resistencia al glifosato (R) se confirmó solo en *C.elata*, donde la población R fue 6.1 veces más resistente en comparación con la población susceptible (S). Además, las plantas R de *C. elata* acumularon 4.6 veces menos shikimato después de la aplicación de glifosato que las plantas S. Las poblaciones de *C.barbata* y *C.ciliata* con o sin historial de aplicación de glifosato mostraron valores similares de DL_{50} y tasas de acumulación de ácido shikimico, lo que demuestra que la resistencia al glifosato no ha evolucionado en estas especies. Las plantas de las poblaciones R y S de *C.elata* difirieron en la absorción y translocación de ^{14}C-glifosato. La población R exhibía 27.3 veces mayor actividad de la 5-enolpiruvil-shikimato-3-fosfato sintasa (EPSPS) que la población S debido a una mutación en el sitio diana correspondiente a una sustitución Pro-106-Ser encontrada en el gen EPSPS. Estos informes muestran la tolerancia innata al glifosato de *C. barbata* y *C. ciliata*, y confirman la resistencia de *C. elata* a este herbicida, mostrando que tanto el sitio no objetivo como los mecanismos del sitio objetivo están implicados en su resistencia al glifosato. Este es el primer caso de resistencia a herbicidas en Cuba.

INTRODUCTION

The use of herbicides is the most common weed control method ever developed (Fernandez-Moreno *et al.,* 2017; Delye, 2013). However, herbicide resistance has caused its quickly cut short. This scenario is the result of evolutionary adaptations a target weed to herbicide applications (Powles and Yu, 2010; Beckie and Harker, 2017). Glyphosate ((N-phosphonomethyl)-glycine) is one of the most widely used herbicides, although it also belongs to the herbicides with many cases of resistance (37 glyphosate-resistant species) (Shaner *et al.,* 2012; Bracamonte *et al.,* 2016; Heap, 2017). This herbicide is non- systemic and non-elective and used in post-emergence, which inhibits the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (EC 2.5.1.19), triggering the catalysis of shikimate-3-phosphate and phosphoenolpyruvate (PEP) to form 5-enolpyruvyl-3-phophate, and important step in the biosynthesis of aromatic amino acids in plants (Franz *et al.,* 1997).

The mechanisms conferring glyphosate resistance are grouped into two major groups (Sammons and Gaines, 2014). Target-site resistance (TSR) can involve EPSPS gene mutations or EPSPS gene amplification. TSR was revealed to be point mutations in the EPSPS gene with substitutions at Thr-102 and Pro-106 (Yu *et al.,* 2015; Alcántara-de la Cruz *et al.,*2016b). Pro-106 substitutions have been found in several weeds (Gonzalez-Torralva *et al.,* 2012; Fernandez *et al.,* 2015; Alarcon-Reverte *et al.,* 2015), conferring low resistance levels to glyphosate in order of 2-to-5 fold, while a double mutation (Thr-102 and Pro-106) increases resistance levels. Gene amplification is an adaptation also conferring resistance to glyphosate (Gaines *et al.,* 2013). The additional EPSPS produced from the amplified gene copies enables the plants to survive higher glyphosate doses (Gaines *et al.,* 2013; Chen *et al.,* 2015; Yu *et al.,* 2015). Non-target-site resistance (NTSR) mechanism results from reduced absorption and/or translocation, increased vacuolar sequestration, and metabolism to non-toxic compounds causing a lesser glyphosate transport via the xylem and phloem to the EPSPS (Delye, 2013). NTSR has being described as the most common mechanism of resistance to glyphosate, which can confer unpredictable resistance (Powles and Yu, 2010). Like TSR, NTSR has been found as mechanism involved in the resistance of many weeds (de Carvalho *et al.,* 2012; Vila-Aiub *et al.,* 2012; Ge *et al.,* 2012; Rojano-Delgado *et al.,* 2012).

The genus *Chloris* Sw. (Poaceae: Chloridoidae) is a C_4 photosynthetic species distributed in tropical and subtropical regions (Molina and Agrasar, 2004). Also, it has been found in semi-arid areas inhabiting semi-natural grasslands and rural habits such as road and waster places (Cerros-Tlatilpa *et al.*, 2015). The genus comprises 50-60 species in both hemispheres (Molina and Agrasar, 2004; Barkworth, 2007). Species of this genus have great economic and ecological importance in the world, because are a source of forage, resist drought, increase soil fertility, require low inversion and can be used as plant cover to protect soil from rain-driven erosion (Ramirez *et al.* 2009). However, some them could be considered as invasive weed species (Cerros-Tlatilpa *et al.*, 2015).

To date in Cuba, there have been practically any herbicide resistant weed studies because of lack of awareness on the issue. This situation is similar to Dominican Republic, in which studies have already begun to be carried out (Bracamonte *et al.,* 2016). Unfortunately, growers have already started to shown in their citrus groves many weeds, which are not controlled at field dose recommended of glyphosate (720 g ae ha^{-1}). Given that weed control strategies in larger commercial fields are absolutely focus on glyphosate applications at post-emergence growth stage, a scientific confirmation has to be necessary. In this way, it could define the species, which are evolving toward to glyphosate resistance, or even tolerant species to this herbicide.

An accurate assessment of taxonomic identity is a prerequisite to address population- and individual plant-based functional studies. This is particularly true in the case of highly diverse genera, for which taxonomic and nomenclatural complexities generally arise, as is the case in *Chloris* (Molina and Agrasar, 2004).

This work was aimed to characterize suspicious glyphosate-resistant populations of three different *Chloris* species from Cuba. Studies were conducted to 1) establish their taxonomical identity based on morphological and molecular analyses, 2) evaluate their resistance/tolerance levels, and 3) determine the mechanisms involved.

MATERIAL AND METHODS

Plant material and experimental conditions

In 2014, our research group (Dr. Rafael De Prado) together with the group of Weed Science of the Ministry of Agriculture of Cuba (Dr. Jorge Cueto) carried out a prospection of *Chloris* species in citrus orchards from central Cuba. These fields had been repeatedly treated with glyphosate (5 L ha^{-1}, 36% w/v) for over 10 years continuous, and sometimes more than one application per year (Cueto, personal communication).

Mature seeds from three suspicious glyphosate-resistant populations of *Clhoris* species (treated=T), were collected in citrus orchards from Arimao (Coordenates) and Ceiba (Coordenates), in Cienfuegos province. Seeds of a population of each species from nearby locations with no known records of exposition to glyphosate were also collected (non-treated=NT).

The seeds were germinated into containers with a substrate of sand/peat (1:2 v/v), covered with parafilm, and placed in a growth chamber at 28/18 °C (day/night) temperature, with 16 h photoperiod (850 μmol m^{-2} s^{-1}) and 80% humidity. Subsequently, the seedlings of each population of the different *Chloris* species were transplanted individually into pots (1 plant per pot) containing the same substrate and placed in the growth chamber under the conditions described above. Further, 20-30 plants per population were placed in a greenhouse until flowering and fruiting.

Morphometric study and taxonomic identity

Different taxonomically relevant morphological traits of inflorescences and caryopses were measured on greenhouse-grown plants of each species of *Chloris* populations. Examined traits on inflorescences were number of racemes, raceme length and spikelet density (number of spikelets per cm of raceme). For the spikelets, we examined length and width of the lower and upper glumes, the number of sterile florets, length of hairs surrounding the callus, length and width of the lemma of the fertile floret (fertile lemma), presence and length of hairs on, or instead adjacent to, the keel and on the margins of fertile lemma, length of the palea of the fertile floret, lemma length and width of the basal sterile floret, lemma length of any additional sterile floret, and presence and length of awns in lemmas. The characters used on caryopses were length,

width, thickness, shape and length of the embryo mark. The shape of caryopses was quantified as the variance of their three dimensions, each relative to length (Thompson *et al.,* 1993). This dimensionless shape index varies between 0 for a perfect sphere and 0.22 for a disk- or needle-shaped item. Based on the above morphological characters, the three pairs of study populations were identified to species level according to Molina and Agrasar (2004), and nomenclature followed IPNI (The International Plant Names Index (2017).

Molecular characterization of the Chloris species by AFLP primer analysis

Twenty-four accessions from the *Chloris* spp. were employed as the study material. Genomic DNA was extracted from fresh young leaves of eight individual plants per species (four T and four NT), using the kit Speedtools Plant DNA Extraction (Biotools). DNA concentration was measured using a Nanodrop ND 1000 spectophotometer. DNA was diluted to a final concentration of 10 ng/μL.

Twelve AFLP primer pairs were used (E36-M48 (E-ACC M-CAC); E36-M60 (E-ACC M-CTC); E37-M49 (E-ACG M-CAG); E38-M50 (E-ACT M-CAT); E40-M61 (E-AGC M-CTG); E35-M49 (E-ACA M-CAG); E36-M49 (E-ACC M-CAG); E35-M61 (E-ACA M-CTG); E40-M62 (E-AGC M-CTT); E32-M60 (E-AAC M-CTC); E33-M50 (E-AAG M-CAT). The reaction mix contained 10 ng template DNA, 2.5 U Taq DNA polymerase, 40 pmol primer, 200 μM dNTPs, 2.5 mM $MgCl_2$, 10 mM Tris-HCl all in a volume of 20 μl. The optimized thermal cycling conditions were 2 min at 94 ºC, followed by 40 cycles of 94 ºC for 25 s, 56 ºC for 25 s, 72 ºC for 25 s and a final extension at 72 ºC for 7 min. AFLP fragments were resolved in 25-cm gels (0.25 mm spacer thickness). Electrophoresis and detection were performed on a two-dye, model 4300 LICOR automated DNA sequencer. Digital AFLP gel images were scored to obtain binary (band presence/absence) data using the SAGA GENERATION 2 software program.

Data clustering was conducted for AFLP with NTSYS-pc-2.2 software (Rohlf 2000) with the use of Jaccard´s coefficients to define unweighted pair-group (UPGMA) dendograms. Principal coordinate analysis (PCA) was also performed with the NTSYS-pc program.

Dose-response assays

Plants of each *Chloris* population were sprayed at the 3-4 leaf growth stage. Glyphosate applications were applied with a laboratory chamber with a laboratory chamber (SBS-060 De Vries Manufactering, Hollandale, MN) equipped with 8002 flat fan nozzle delivering 200 L ha^{-1} at 250 KPa at the height of 50 cm. The following glyphosate (Roundup®, 360 g ae L^{-1} as isopropylamine salt) rates were used: 0, 62.5, 125, 250, 500, 1000, 2000, 3000, and 4000 g ae ha^{-1}. The experiment was design using nine replications per rate and was repeated twice. Plants were cut down at the soil surface 21 days after application (DAT).

Shikimic accumulation assay

Fifty mg of fresh tissue (4 mm leaf disks) were harvested from the youngest fully expanded leaf at the 3-4 leaf growth stage from 15 plants per population. Shikimic acid accumulation was determined according to Hanson *et al.* (2009). The glyphosate concentrations used were: 0, 500 and 1000 µM. Sample absorbance was measured in a Beckman DU-640 spectrophotometer at 380 nm. The test was performed in triplicate on five treated and non-treated plants of each biotype in a completely random design. Results were expressed in mg of shikimic acid g^{-1} fresh tissue.

^{14}C-glyphosate absorption and translocation

This study was carried out in the two *C.elata* populations.^{14}C-glyphosate (American Radiolabeled Chemicals, Inc., USA) was added to the commercial herbicide to prepare a solution with a specific activity of 0.834 kBq μL^{-1}. The final glyphosate concentration corresponded to 360 g ae ha^{-1} in 200 L ha^{-1}. Plants were treated (0.834 kBq $plant^{-1}$) at 24, 48, 72 and 96 h after treatment (HAT). Five plants per populations at each time evaluated in a completely random design were handled according to Fernández-Moreno *et al.* (2017). Radioactivity was analyzed by liquid scintillation spectrometry (LSS) in a Beckman LS 6500 scintillation counter (Beckman Coulter Inc. Fullerton, USA) during 10 min per sample. Percentage of ^{14}C-glyphosate absorbed was expressed as [kBq in combusted tissue / (kBq in combusted tissue + kBq in leaf washes)] × 100.

Translocation of ^{14}C-glyphosate in plants of the two *C. elata* populations was visualized using a phosphor imager (Cyclone, Perkin-Elmer, Waltham, MA, USA).

Glyphosate metabolism

Six plants by each *C. elata* population at 3-4 leaf growth stage, were treated with 300 g ae ha^{-1} of glyphosate (as described in the dose-response assays) in a completely randomized design. Untreated plants were used as controls. Leaf tissues were washed with distilled water at 96 HAT, flash-frozen in liquid nitrogen, and stored at −40 °C until use. Following the methodology described by Rojano-Delgado *et al.* (2010), glyphosate and its metabolites (aminomethyl phosphonate (AMPA), glyoxylate, sarcosine and formaldehyde) were determined by reversed polarity capillary electrophoresis using a 3D Capillary Electrophoresis Agilent G1600A instrument equipped with a diode array detector (DAD, wavelength range 190–600 nm). Standard compounds used (glyphosate, AMPA, sarcosine, formaldehyde, and glyoxylate), were provided by Sigma-Aldrich, Spain. Glyoxylate naturally produced (untreated plants) was subtracted from the average of glyoxylate produced from glyphosate metabolism (treated plants) for each population.

EPSPS enzyme activity assays

Leaf tissue of the *C. elata* populations (three samples of 5 g each) were ground to fine powder in liquid nitrogen a chilled mortar. The enzyme activity was axtracyted according to the protocol described by Sammons *et al.* (2007). The basal EPSPS activity in the extract was measured using a Modified Lowry Kit for Protein Determination (Sigma-Aldrich, Madrid, Spain) in accordance with the manufacturer´s instructions. The specific EPSPS activity was determined using the EnzCheck Phosphate Assay Kit (Invitrogen, Carlsbad, CA) following the manufacturer´s instructions, to determine the inorganic phosphate release. The glyphosate concentrations used were: 0, 0.1, 1, 10, 100, and 1000 μM. The EPSPS activity was measured during 10 minutes at 360 nm in a spectrophotometer (Beckman DU-640) to determine the amount of phosphate (μmol) released μg of total soluble protein $(TSP)^{-1}$ min^{-1} and expressed as a percentage with respect to the control (without glyphosate). The experiment was repeated three times for each samples.

EPSPS gene sequence

Young tissue (100-200 mg) was collected of ten plants of each *C. elata* population and stored at -80 ºC for RNA extraction. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA

was then treated with TURBO DNase (RNase-Free; Ambion, Warrington, UK) to eliminate any DNA contamination. cDNA synthesis was carried out from 2 μg of total RNA using a M-MLV (Moloney Murine Leukemia Virus) Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) in combination with oligo $(dT)_{12-18}$ and random nonamers (Amersham Biosciences, Amersham, UK) according to the manufacturer's instructions. To amplify the EPSPS gene, primers previously designed by Perez-Jones *et al.* (2007) (forward: 5′ AGCTGTAGTCGTTGGCTGTG 3′; reverse: 5′ GCCAAGAAATAGCTCGCACT 3′), and by Carvalho *et al.* (2012) (forward: 5' TAGTACAGCCAAAAGGGCAGTC-3'; reverse: 5 'GCCGTTGCTGGAGGAAATTC 3') were used. These primers expand a 120-bp fragment of the EPSPS gene that contains the mutation site described as conferring resistance to glyphosate. The PCR reactions were carried out using cDNA from 50 ng of total RNA, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 μM of each primer, 1× buffer, and 0.625 units of a 100:1 enzyme mixture of non-proofreading (*Thermus thermophilu*s) and proofreading (*Pyrococcus furiosus*) polymerases (BIOTOOLS, Madrid, Spain) in a final volume of 25 μL. All PCR reactions were in duplicate and cycling conditions were: 94 °C 3 min, 35 cycles of 94 °C 30 s, 55 °C 30 s and 72 °C 1 min; and a final extension cycle of 72 °C 10 min. An aliquot of the PCR product was loaded in a 1 % agarose gel to check the correct band amplification. The rest of the PCR product was then purified using ExoSAP-IT® for PCR Product Clean-Up (USB, Ohio, USA) as indicated by the manufacturers. Five purified PCR products per population were sequenced (STAB VIDA, Caparica, Portugal).

Statistical analysis

Dose-response and EPSPS enzyme activity data were subjected to non-linear regression analysis to find out the amount of glyphosate needed to reduce the fresh weight (GR_{50}), mortality (LD_{50}), and to inhibit EPSPS activity (I_{50}) by 50% of each *Chloris* populations using the three-parameter log-logistic function: $y = ([(d) / 1+(x/g)^b])$ where y is, depending on the analysis, the above ground fresh weight, survival, or EPSPS-activity expressed as the percentage of the non-treated control, d is the parameter corresponding to the upper asymptote, b is the slope, g is the GR_{50}, LD_{50}, or I_{50}, and x (independent variable) is the glyphosate rate. Regression analyses were conducted using the *drc* package with program R version 3.2.5 (Ritz *et al.*, 2015). Resistance indexes (RI=R/S) were computed as R-to-S GR_{50}, LD_{50} or I_{50} ratios.

Analysis of variance (ANOVA) was conducted to test for differences between populations in the different assays. When needed, differences between means were separated using the Tukey HSD test at $P<0.05$. Model assumptions of normal distribution of errors and homogeneous variance were graphically inspected. ANOVAs were conducted using the Statistix (version 9.0) (Analytical Software, USA) software.

RESULTS

Morphometric study and taxonomic identity

Based on the examined morphological traits, the populations studied were identified as *C. ciliata* Sw., *C. elata* Desv. and *C. barbata* Sw. Only the latter is an annual species, and it can be easily separated from the two other species by its long-awned lemmas, the glabrous keel of the fertile lemma and the presence instead of hairs flanking the keel. In addition, caryopses of *C. barbata* were clearly more elongated in shape than those of the remaining species, as indicated by the higher values of the seed shape index. Distinctive traits of *C. ciliata* include a low number of racemes in the inflorescences, more than two sterile florets per spikelet, long keel hairs and relatively short awns. Compared to the two other species, racemes of *C. elata* plants were consistently longer and their fertile lemmas were shorter in length, showing much longer marginal hairs. In addition, the embryo mark in caryopses was neatly shorter in this latter species (Table 1).

Table 1. Comparison of morphological traits from inflorescences and caryopses for the three pairs of studied *Chloris* populations, and their taxonomic identity at the species level

Species		*C. ciliata* T	*C. ciliata* NT	*C. elata* T	*C. elata* NT	*C. barbata* T	*C. barbata* NT
Number of racemes composing inflorescences		5 (5-7)	5 (4-5)	17-28	11-27	13 (11-15)	12-18
Racemes length		54.6 ± 6.8	76.0 ± 12.0	117.0 ± 20.9	96.0 ± 16.9	71.2 ± 8.5	73.0 ± 9.2
Number of spikelet per cm raceme		14 (14-14)	14 (12-14)	11 (9-11)	14 (12-15)	12 (12-13)	13 (12-13)
Number of sterile florets in spikelets		3 (3-4)	4 (3-4)	2 (2-2)	2 (2-2)	2 (2-2)	2 (2-2)
Length of hairs surrounding spikelet callus		0.66 ± 0.21	0.79 ± 0.12	0.18 ± 0.03	0.40 ± 0.06	0.95 ± 0.08	0.97 ± 0.12
Fertile floret, lemma	Length	2.68 ± 0.19	2.65 ± 0.18	1.98 ± 0.11	1.87 ± 0.10	2.63 ± 0.12	2.54 ± 0.10
	Max width	2.14 ± 0.19	2.18 ± 0.20	1.36 ± 0.09	1.56 ± 0.10	1.18 ± 0.17	1.13 ± 0.14
	Hairy (H)/glabrous (G) keel	H	H	H	H	G	G
	Length of keel hairs	1.20 ± 0.15	1.30 ± 0.15	0.67 ± 0.05	0.70 ± 0.06	-	-
	Length of hairs flanking keel	-	-	-	-	0.57 ± 0.08	0.58 ± 0.14
	Length of marginal hairs	1.13 ± 0.15	1.42 ± 0.26	2.22 ± 0.16	2.09 ± 0.10	1.40 ± 0.13	1.40 ± 0.06
	Awn length	1.63 ± 0.26	1.71 ± 0.35	3.14 ± 0.36	2.53 ± 0.27	6.97 ± 0.60	5.53 ± 1.08
Fertile floret, palea	Length	2.44 ± 0.14	2.55 ± 0.20	1.82 ± 0.09	1.58 ± 0.09	2.45 ±0.14	2.35 ± 0.23
Basal (first) sterile floret, lemma	Length	1.66 ± 0.14	1.73 ± 0.18	1.09 ± 0.08	1.05 ± 0.05	1.38 ± 0.12	1.37 ± 0.05
	Max width	2.15 ± 0.21	2.23 ± 0.23	0.92 ± 0.10	0.97 ± 0.08	1.27 ± 0.28	1.33 ± 0.08
	Awn length	1.58 ± 0.26	1.72 ± 0.23	2.83 ± 0.15	2.22 ± 0.29	6.37 ± 1.23	5.33 ± 1.89
Second sterile floret, lemma	Length	1.14 ± 0.17	1.08 ± 0.15	0.50 ± 0.05	0.53 ± 0.04	1.29 ± 0.09	1.33 ± 0.08
	Awn length	A	A	A	A	5.22 ± 0.81	3.82 ± 0.88
Glumes	Upper, length	2.57 ± 0.17	2.78 ± 0.07	3.10 ± 0.06	3.25 ± 0.20	2.82 ± 0.08	2.78 ± 0.12
	Upper, width	1.00 ± 0.19	0.92 ± 0.13	0.53 ± 0.05	0.45 ± 0.14	0.60 ± 0.06	0.55 ± 0.05
	Lower, length	1.75 ± 0.15	1.73 ± 0.08	2.07 ± 0.13	2.03 ± 0.14	1.78 ± 0.08	1.80 ± 0.06
	Lower, width	0.83 ± 0.10	0.78 ± 0.04	0.73 ± 0.08	0.80 ± 0.06	0.58 ± 0.08	0.45 ± 0.05
Caryopsis	Length	1.43 ± 0.08	1.35 ± 0.12	1.12 ± 0.04	1.03 ± 0.05	1.45 ± 0.05	1.40 ± 0.00
	Width	0.70 ± 0.04	0.58 ± 0.09	0.57 ± 0.04	0.60 ± 0.00	0.45 ± 0.03	0.43 ± 0.03
	Thickness	0.58 ± 0.04	0.53 ± 0.07	0.45 ± 0.03	0.47 ± 0.03	0.33 ± 0.03	0.31 ± 0.02
	Length of the embryo mark	0.83 ± 0.08	0.77 ± 0.09	0.59 ± 0.07	0.62 ± 0.13	0.93 ± 0.03	0.90 ± 0.05
	Seed shape index [1]	0.069 ± 0.008	0.078 ± 0.001	0.068 ± 0.005	0.055 ± 0.008	0.121 ± 0.004	0.122 ± 0.005

[1] Variance of the three dimensions, each relative to length. Means and standard deviations are given for quantitative traits, and modal (if any), minimum and maximum values for qualitative traits. Sample size $n = 6$. Linear dimensions are given in mm. A, absent.

Molecular characterization of the genus Chloris

The cluster analysis using UPGMA methods classified *Chloris* populations in two major groups (I and II), thus providing complementary information to the morphologically-based analysis. Group I contained all samples of *C. ciliata* whereas Group II consisted of two subgroups, II-1 including *C. elata*, and II-2 including *C. barbata*. It is worth noting that while the cluster analysis did not separate T and NT populations neither of *C. ciliata* nor *C. barbata*, it clearly separated the T and NT populations of *C. elata* (Figure 1).

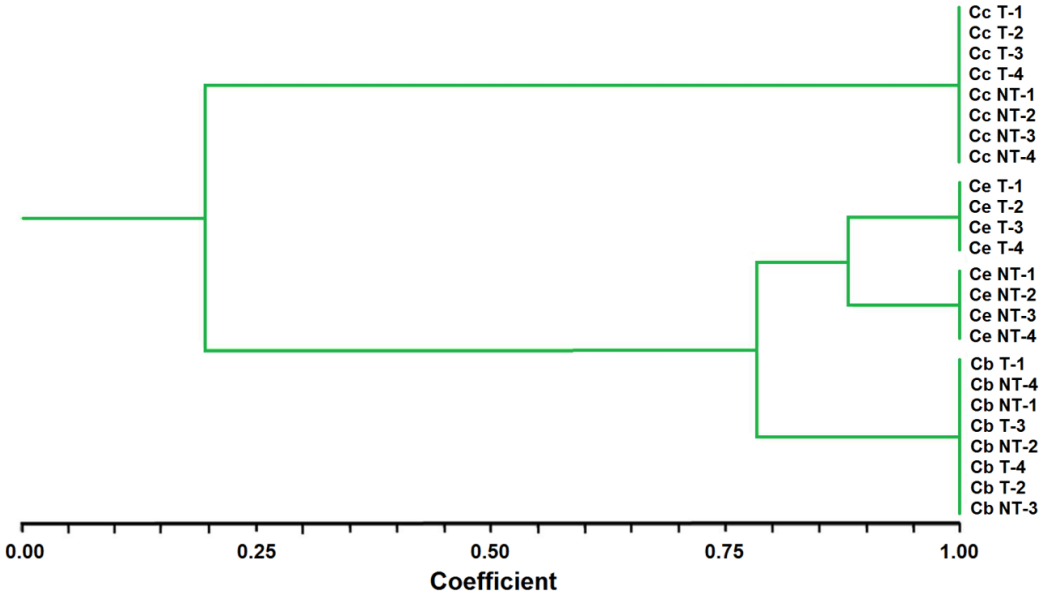

Figure 1. Genetic similarities among *Chloris* species after UPGMA analysis performed with AFLP market data. Ce= *Chloris elata*; Cb= C. *barbata*; and Cc= *C. ciliata.* T= plants with glyphosate history applications; and NT= non-treated plants.

Dose-response assays

The fresh weight reduction by 50% (GR_{50}) for the NT and T populations of *C. elata* was achieved at 88.3 and 542.1 g ae ha^{-1}, respectively, i.e. T populations was 6.14-fold more resistance with respect to the NT one. The survival values exhibited 15.02-fold resistance for the T with respect to the NT of *C. elata* populations. Contrary, GR_{50} and LD_{50} values in both *C. barbata* and *C. ciliata* species were not different compared with their respective populations T and NT (Table 2, Figure 2).

Table 2. Glyphosate rates required for 50 % reduction fresh weight (GR_{50}) and survival (LD_{50}) expressed as percentage of the mean untreated control of *Chloris* species.

Species	Status[a]	GR_{50} (g ae ha^{-1})	RI[b]	*P*	LD_{50} (g ae ha^{-1})	RI[b]	*P*
C. elata	T	542.1 ± 31.3	6.1	0.001	2277.7 ± 245.1	15.0	0.001
	NT	88.3 ± 4.8			151.6 ± 24.8		
C. barbata	T	217.2 ± 19.6	1.1	0.241	889.4 ± 71.5	1.1	0.382
	NT	198.9 ± 21.9			820.2 ± 79.0		
C. ciliata	T	263.1 ± 19.2	1.1	0.159	912.7 ± 68.6	1.1	0.297
	NT	231.3 ± 37.4			875.2 ± 59.3		

± Standard error (n = 10). [a] Status: T= populations with glyphosate history applications; and NT= non-treated populations with glyphosate. [b] RI = Resistance index (R/S) calculated using the corresponding ED_{50}, or LD_{50} values of the resistant populations respect to the susceptible one.

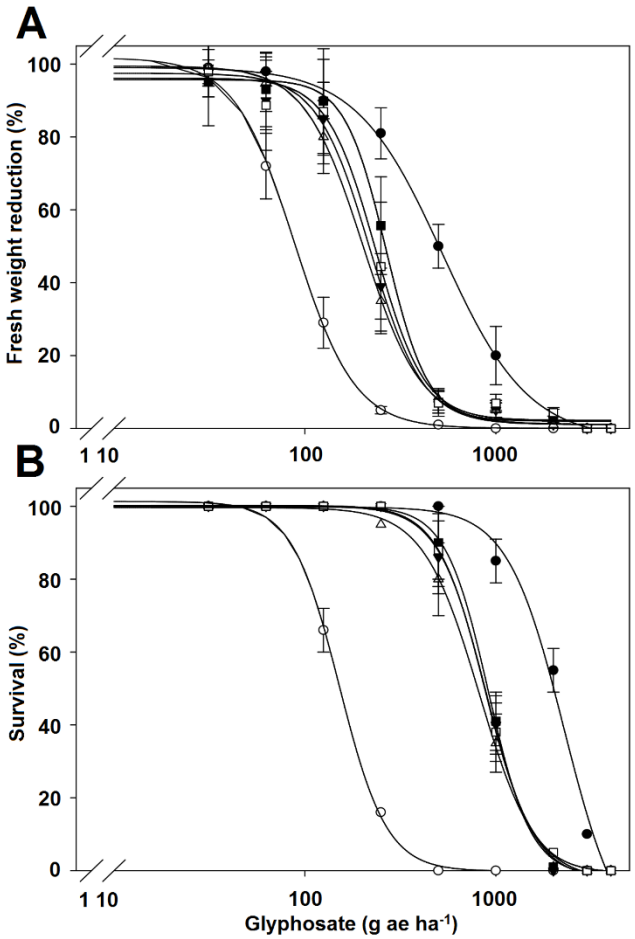

Figure 2. Glyphosate A) dose-response on fresh weight reduction and B) % survival expressed as percentage respect to untreated control of the treated (T) (●) and non-treated (NT) (○) populations of *Chloris elata*; T (▼) and NT (Δ) populations of *C. barbata*; and T (■) and NT (□) populations of *C. ciliata*. Vertical bars are ± standard errors (n= 9).

Shikimic acid accumulation

No significant differences were found between exposition of leaf disks to 500 or 1000 µM of glyphosate. At 1000 µM, NT population of *C. elata* presented 4.6 fold more

shikimic than T population. However, *C. barbata* and *C. ciliata* obtained similar accumulation between T and NT populations with 1.13 and 1.07 fold shikimic acid, respectively (Figure 3).

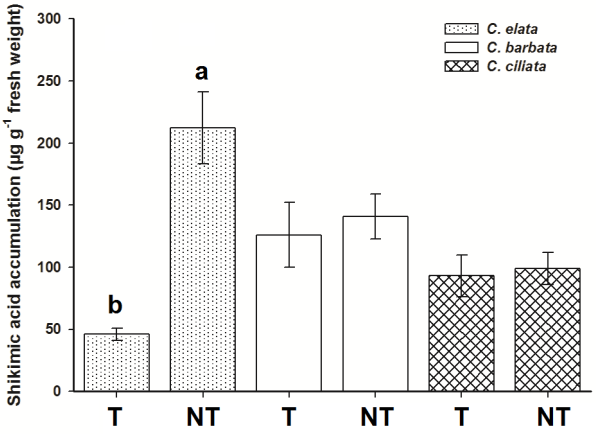

Figure 3. Shikimic acid accumulation in *Chloris* spp. plants at 1000 µM glyphosate concentration. T= plants with glyphosate history applications; and NT= non-treated plants. Vertical bars are ± standard errors (*n*= 5).

It was determined that *C. barbata* and *C. ciliata* as exhibiting natural tolerance to glyphosate, and the T and NT populations of *C. elata* were renamed as resistant (R) and susceptible (S) to glyphosate, respectively. The following assays, we focused in *C. elata.*

Absorption, translocation and visualization of ^{14}C-glyphosate

Total ^{14}C-glyphosate recovery was 93.2 and 94.3% for R and S populations of *C. elata*, respectively (data not shown). ^{14}C-glyphosate absorption increased slowly in the first 72 HAT, but amount absorbed was was higher in S *C. elata* population than in R one. The maximum glyphosate absorption was observed between 72 to 96 HAT, being the two-fold in the S population than in the R one (Figure 4).

In both populations, ^{14}C-glyphosate levels in treated leaves declined from 24 to 96 HAT, being the rate of movement out of the treated leaf greater and faster in the S population than in R one. At 96HAT, the glyphosate translocation in shoots and roots was two-fold in S population compared to the R one (Figure 4B, 4C).

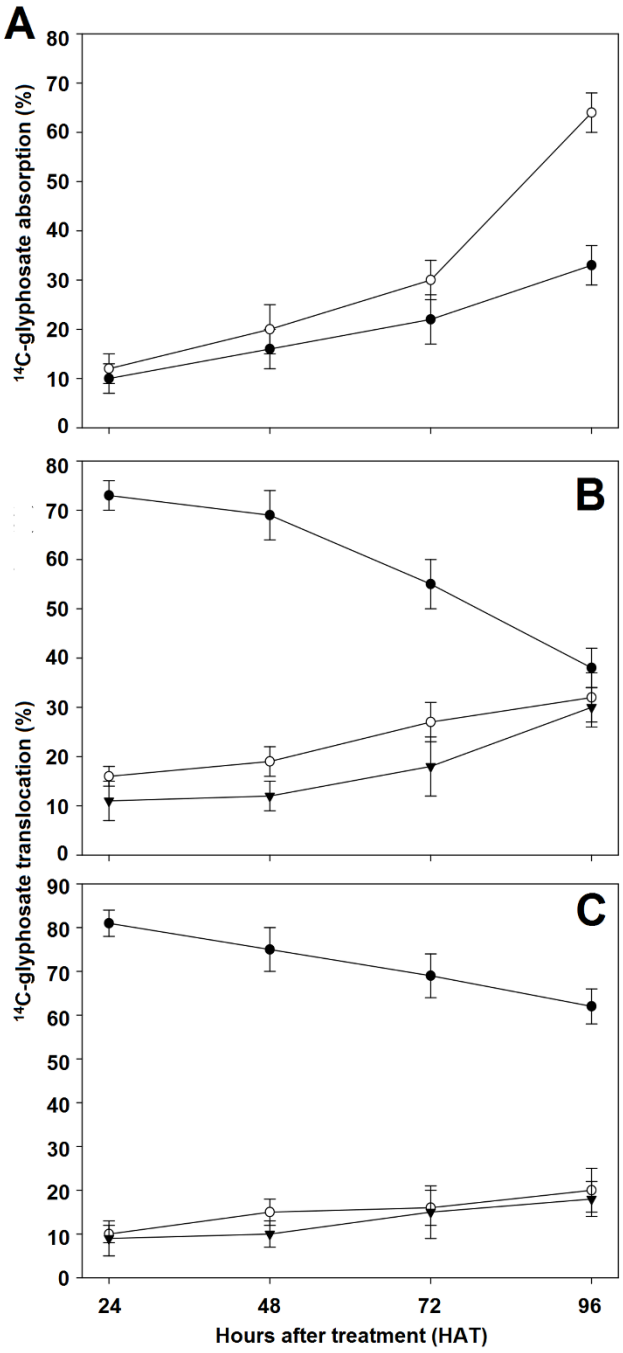

Figure 4. ^{14}C-glyphosate absorption in susceptible (S) and resistant (R) *Chloris elata* populations. A) ^{14}C-glyphosate absorption in S (○) and R (●) plants. B) ^{14}C-glyphosate translocation in the S population, and C) in the R population from 24 to 96 hours after glyphosate treatment. Treated leaf (●), remaining shoot tissue (○), and roots (▼). Vertical bars are ± standard errors (*n*= 5).

The ^{14}C-glyphosate visualization by phosphor imaging revealed differences in the distribution between S and R populations of *C. elata.* There was a difference in the translocation of glyphosate from treated leaves to shoots and roots, and the S population translocated higher amounts of ^{14} C-glyphosate compared to R population (Figure 5).

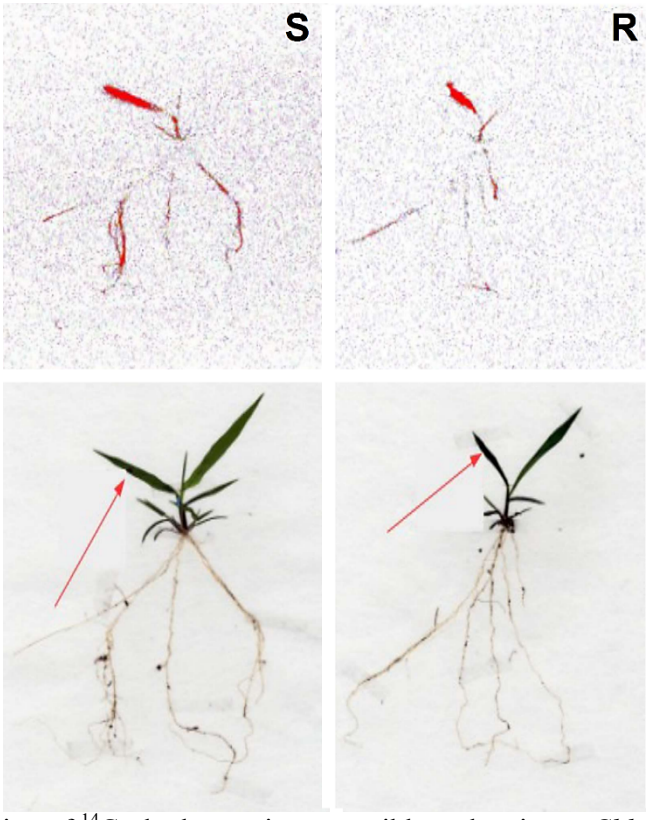

Figure 5. Translocation of ^{14}C-glyphosate in susceptible and resistant *Chloris elata* plants at 96 hours after application. The highest concentration of ^{14}C-glyphosate is highlighted in red. Arrows indicate the treated leaf.

Glyphosate metabolism

For glyphosate absorbed into R and S *C. elata* populations, much of the herbicide was unaltered in plants by 96 HAT. At this time, 89.4 and 91.0% of applied herbicide remained as glyphosate in plants of R and S populations, respectively. The levels of AMPA were 7.2 and 6.4%, while glyoxylate levels attained 3.4% and 2.6% in R and S plants, respectively. For both AMPA and glyoxylate, these differences between R and S populations were non-significant (P= 0.8741 for AMPA, and P=0.6318 for glyoxylate).

EPSPS enzyme activity assays

The R population was 27.3-fold resistance than the S one. The basal enzyme activity showed no differences between populations with 0.0987 to 0.0937 mmol mg^{-1} TPS^{-1} min^{-1}for the R and S populations, respectively (Figure 6).

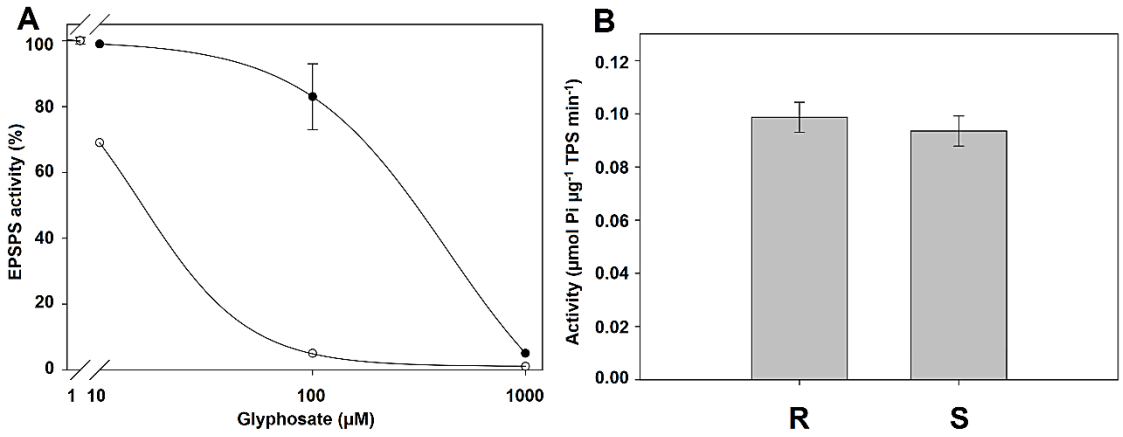

Figure 6. A) EPSPS enzyme activity expressed as percentage of the untreated control in leaf extracts of plants from glyphosate-susceptible and -resistant *Chloris elata* plants. B) Basal EPSPS activity for *C. elata* populations. Vertical bars are ± standard errors (*n*= 3).

Sequencing of the EPSPS gene

A total of 120 bp of the EPSPS gene of R and S *C. elata* populations were sequenced. The fragments were aligned and numbered based on a published EPSPS sequence of *L. virgata* (GenBank: KX425854) (Alcántara-de la Cruz *et al.,* 2016c). Protein alignment of the predicted EPSPS fragments from R and S populations of *C. elata* showed 91.6 and 92.5% of protein similarity, respectively, to that of *L. virgata*. The R population of *C. elata* showed an amino acid substitution at position 106 consisting of a Proline to Serine. The substitution consisted in the presence of the TCG (Serine) codon instead of CCG (Proline) (Figure 7).

Position	99	100	101	**102**	103	104	105	**106**	107	108	109
Consensus sequence	AAT	GCA	GGA	ACC	GCT	ATG	CGC	**CCG**	TTG	ACC	GCT
L. virgata (KX425854)	AAC	GCT	GGA	ACT	GCG	ATG	CGC	**CCA**	TTG	ACG	GCT
Amino acid translation	Asn	Ala	Gly	Thr	Ala	Met	Arg	**Pro**	Leu	Thr	Ala
C. elata S	Asn	Ala	Gly	Thr	Ala	Met	Arg	**Pro**	Leu	Thr	Ala
C. elata R	Asn	Ala	Gly	Thr	Ala	Met	Arg	**Ser**	Leu	Thr	Ala

Figure 7. Partial alignment of nucleotides and amino acid sequences of EPSPS genes between glyphosate-susceptible and -resistant *Chloris elata* populations and *Leptochloa virgata* (GenBank: KX425854). The yellow color indicates nucleotide changes among species. The green color indicates an amino acid substitution at 106 position from Proline (CCG) to Serine (TCG). Box includes the 102 (blue) and 106 (orange) positions (amino acid number based on the start codon (ATG) of *Arabidopsis thaliana* [GenBank: KX425854 EPSPS sequence), corresponding to point mutations associated for conferring glyphosate resistance.

DISCUSSION

The tree *Chloris* populations from citrus orchards of Cuba were identified as *C. barbata, C. ciliata* and *C. elata*. The number of species of *Chloris* recognized by different authors in Mexico and in the nearby Caribbean Islands is variable (Barkworth, 2007; Cerros-Tlatilpa *et al.*, 2015). In these regions, the most frequent species are *C. ciliata, C. elata, C. barbata* and *C. virgata* (Cerros-Tlatilpa *et al*., 2015). In Cuba, twelve species of *Chloris* genus have been found (Catutsus-Guerra, 2002).

The AFLP-based classification of *Chloris* populations revealed molecular-based relationships between three basic entities, which closely matched the morphology-based identification of three different species. The selected AFLP markers can be useful candidates in the pursuit of disentangling phylogenetic relationships among *Chloris* species. In addition, these AFPL markers separated inside *C. elata* both T and NT populations. Thus these markers can be an adequate, fast tool to detect resistance to glyphosate in populations of this *Chloris* species (Beckie *et al*., 2000).

An important question to consider with *Chloris* species is the innate tolerance to glyphosate of the genus. Depending on the species studied, the LD_{50} (% survival plant) values can vary between 515 and 703 g ae ha^{-1} (Ngo *et al.*, 2017a, b). These values are lower than those we have found for *C. barbata* and *C. ciliata*, including the populations never exposed to this herbicide, demonstrating an innate tolerance to glyphosate in these two species. Similar results were described for *C. polydactyla* from Brazil, where even some accessions with no history applications presented lower susceptibility to glyphosate, than those accessions with history applications (Barroso *et al.,* 2014). Innate tolerance has been well studied in grass weeds (Fernandez-Moreno *et al.*, 2016), and leguminous species (Rojano-Delgado *et al.*, 2012; Cruz-Hipolito *et al.*, 2009, 2011; Alcántara-de la Cruz *et al.*, 2016a; Mao *et al.*, 2016). The mechanism proposed is a lack of ^{14}C-glyphosate absorption and/or translocation in tolerant plants compared to susceptible ones.

Chloris elata has a different profile than *C. barbata* and *C. ciliata*, and its GR_{50} and LD_{50} values demonstrate a clear quantitative difference between those plants harvested from fields T compared to those plants from NT fields. The lower shikimic accumulation by the T *C. elata* population (4.9 times) than NT one, showed the great

resistance level of this species, similar to other *Chloris* species like *C. elata* (5.4) from Brazil (Brunharo *et al.*, 2016), and *C. virgata* (2.0-9.7) and *C. truncata* (2.4-8.7) from Australia (Ngo *et al.*, 2017a, b). When glyphosate is applied via foliar, the EPSPS enzyme is inhibited and there is a rapid accumulation of shikimate (Shaner *et al.*, 2005). The glyphosate amount in NT population of *C. elata* determined the inhibition of EPSPS and rapid shikimate accumulation demonstrating the high susceptibility (S) of this population. Therefore, the population T of *C. elata* was characterized as resistant (R) to glyphosate, and the populations T and NT of *C. barbata* and *C. ciliata* as tolerant to this herbicide. These results are reflected in those obtained in dose response assays. For this reason, we just continue to study the glyphosate resistance mechanisms only in the case of *C. elata*.

To date, few cases of reduced glyphosate absorption and/or translocation have been studied as a mechanism of resistance in the genus *Chloris,* and the results are contradictory. ^{14}C-glyphosate studies on *C. virgata* and *C. truncata* do not show significant differences in the absorption and subsequent translocation of the herbicide, and that resistance was determined by mechanisms within the target site (Ngo et al 2017a, b). However, another study on *C. elata* shows that lower glyphosate absorption and translocation in the R population are the only mechanisms involved in its glyphosate resistance (Brunharo *et al.*,2016). In our case the R *C. elata* population collected in Cuba shows a resistance mechanism similar to that previously found for the species. Thus, the ^{14}C-absorption and translocation is higher in the S population than the R one. These results suggest that less absorption and translocation contributed in the resistance to glyphosate R *C. elata* plants.

Glyphosate metabolism has not thus far been identified as a major mechanism of resistance in plants, but is likely the result of plants not succumbing to glyphosate because of expression of another resistance mechanism (Sammons and Gaines, 2014; Fernandez-Moreno *et al.*, 2017). Only in a few cases, metabolism has been demonstrated to play as secondary mechanism in glyphosate resistance, because in these cases other main mechanisms are involved (Bracamonte *et al.*, 2016). Our research substantiates that of other studies, with greater than 90% of absorbed glyphosate remaining unaltered in R and S plants of *C. elata*. It is likely that the ability of grass weeds to metabolize glyphosate is diminished once EPSPS is inhibited (Gonzalez-

Torralva *et al.*, 2012; Fernandez *et al.*, 2015). Considering the small extent of glyphosate metabolism, the significance of this are not likely biologically meaningful in the resistance to glyphosate in *C. elata*.

The I_{50} values were significant different between the *C. elata* populations. The R population exhibited a high resistance level compared to S population. Results with high I_{50} values and low shikimic acid values, as it has already been explained and demonstrated in other studies, are associated with alterations in the gene encoding the herbicide target protein (Sammons and Gaines, 2014; Yu *et al.,* 2015). Then, TSR mechanism could be involved in the resistance to this species. Similar results have been shown in other weed species including *Leptochloa virgata* (Alcantara de la Cruz *et al.,* 2016c), *Lolium multiflorum* (Salas *et al.,* 2015) and *L. rigidum* (Fernandez *et al.,* 2015). In these cases, higher I_{50} values, as well as a higher basal activity of EPSPS were found in the resistant populations compared to the susceptible ones. It was thought as overexpression of EPSPS playing as resistance mechanism (Ngo *et al.,* 2017a). However, there were not significant differences in the basal activity of EPSPS between R and S populations of *C. elata*, precluding the involvement of such a mechanism.

The EPSPS sequence alignment showed only a mutation point at position Pro-106-Ser in the GR *C. elata* population. Four substitution in this genomic EPSPS position (Pro-106-Ser, Pro-106-Thr, Pro-106-Ala and Pro-106-Leu), has been reported in mono- and dicotyledonous weeds endowing resistance to glyphosate (Sammons and Gaines, 2014). A changing at this point mutation to a different amino acid causes a structural change in the target site, shifting other amino acids towards the inhibitor by reducing the available space (Healy-Fried *et al.,* 2007). These explain the resistance at molecular level of the R population of *C. elata.* Some grassweed species which have shown a mutation at Pro-106 position are: *C. virgata* (Ngo *et al.,* 2017a)*, Echonoclhoa colona* (Alarcón-Reverte *et al.,* 2015), *L. virgata* (Alcántara-de la Cruz *et al.,* 2016c), *L. rigidum* (Fernandez *et al.,* 2015) and *Poa annua* (Cross *et al.,* 2015), among others.

CONCLUSIONS

Morphological and molecular-basis allowed the identification of the three *Chloris* species collected in citrus orchards from central Cuba. *Chloris barbata* and *C. ciliata*

were characterized as being innate tolerant to glyphosate, and *C. elata* as resistance to this herbicide. The latter species involves non-target site (reduced absorption and translocation) and target site (Pro-106-Ser mutation) resistance mechanisms to resist against to glyphosate.

These results confirm the first case of herbicide resistance in Cuba, and evidences strongly that species of the *Chloris* genus can be either resistant or tolerant to glyphosate, supporting the previous reports of both glyphosate statuses in this genus.

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CAPÍTULO IV

From tolerance to resistance: Mechanisms governing the differential response to glyphosate in *Chloris barbata*

Bracamonte, E., Silveira, H. M. D., Alcántara-de la Cruz, R., Domínguez-Valenzuela, J. A., Cruz-Hipolito, H. E., & De Prado, R. (2018). From tolerance to resistance: mechanisms governing the differential response to glyphosate in Chloris barbata. ***Pest management science*****,** ***74*****(5), 1118-1124.**

ABTRACT

Species of the genus *Chloris* have a certain level of natural tolerance to glyphosate. Variable susceptibility to glyphosate and the mechanism(s) governing tolerance/resistance to this herbicide were characterized into two putative glyphosate-resistant *Chloris barbata* populations (R1 and R2), collected in Persian lime orchards from Colima State, Mexico, where received glyphosate (720 g ae ha^{-1}) applications 7-year (3-4 times per year). The resistant (R) populations were compared to one non-treated (referred as S) population. The amounts of glyphosate to reduce fresh weight and cause mortality by 50% of R populations were 4.2-6.4 times higher than the S population. The latter one accumulated from 4.3-5.2 times more shikimic acid than the R populations. There were no differences in ^{14}C-glyphosate uptake between R and S *C. barbata* populations, but the R plants translocated at least 12% less herbicide to the rest of plant and roots 96 hours after treatment. Insignificant amounts of glyphosate were metabolized to AMPA and glyoxylate in both R and S plants. The 5-enolpyruvylshikimate-3-phosphate synthase gene of the R *C. barbata* populations contained the Pro-106-Ser mutation, giving them a resistance up to 12-14.7 times greater at the target site in comparison with the S plants. The continuous applications of glyphosate increased tolerance and induced resistance in *C. barbata.* The Pro-106-Ser mutation governs the resistance to glyphosate of the R1 and R2 *C. barbata* populations, but the impaired translocation could be contributing to the resistance. These results confirm the first case of glyphosate resistance evolved in this species.

RESUMEN

Las especies del género *Chloris* tienen un cierto nivel de tolerancia natural al glifosato. La variable susceptibilidad al glifosato y el (los) mecanismo (s) que gobiernan la tolerancia / resistencia a este herbicida se caracterizaron en dos poblaciones putativas de *Chloris barbata* resistentes al glifosato (R1 y R2), recolectadas en huertos de limas del estado de Colima, México, donde recibieron aplicaciones de glifosato (720 g ae ha^{-1}) durante 7 años (3-4 veces por año). Las poblaciones resistentes (R) se compararon con una población no tratada (referida como S). Las cantidades de glifosato para reducir el peso fresco y causar mortalidad en un 50% de las poblaciones R fueron 4.2-6.4 veces más altas que la población S. El último acumuló de 4.3 a 5.2 veces más ácido shikímico que las poblaciones R. No hubo diferencias en la captación de ^{14}C-glifosato entre las poblaciones R y S *C. barbata*, pero las plantas R translocaron al menos un 12% menos de herbicida al resto de las plantas y las raíces 96 horas después del tratamiento. Cantidades insignificantes de glifosato se metabolizaron en AMPA y glicoxilato en plantas R y S. El gen 5- enolpyruvylshikimate-3-fosfato sintasa de las poblaciones de *C. barbata* contenía la mutación Pro-106-Be, dándoles una resistencia a 12-14.7 veces mayor en el sitio diana en comparación con las plantas S. Las aplicaciones continuas de glifosato aumentaron la tolerancia y la resistencia inducida en *C. barbata*. La mutación Pro-106-Ser regula la resistencia al glifosato de las poblaciones R1 y R2 de *C. barbata*, pero la translocación alterada podría estar contribuyendo a la resistencia. Estos resultados confirman el primer caso de resistencia al glifosato desarrollado en esta especie.

INTRODUCTION

Mexico is the second largest producer and main exporter of Persian lime **(***Citrus latifolia* Tan.).[1] Veracruz (Gulf of Mexico) is the main producer of this citrus fruit, but to cover the large volume of exports, its production has been extended to states with similar environmental characteristics, such as Colima, Guerrero,Michoacán and Oaxaca (Pacific Coast).[2] Tropical rainfall conditions allow continuous Persian lime production all year round,but also favor the emergence of weeds, such as *Bidens pilosa***,**[3] *Eleusine indica*[4] and *Leptoclhoa virgate***,**[5] which have been identified as being resistant to glyphosate. They do not have a great impact on yield, but weeds can make cultivation difficult.[6] Therefore, the presence of weeds can have a significant impact on production costs if they remain uncontrolled.[7]

Weed management in citrus orchards in Mexico includes mechanical, chemical (mainly glyphosate-based herbicides)[8] and combined methods.[7] Although glyphosate [(**N**-phosphonomethyl) glycine] belongs to the herbicide group with the greatest increase in resistance cases in recent years, it is the most widely used non-selective and systemic herbicide.[9,10] This herbicide deactivates the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (EC 2.5.1.19) enzyme in the shikimic pathway by interrupting the catalysis of shikimate-3-phosphate and phosphoenolpyruvate (PEP) to form 5-enolpyruvylshikimate-3-phosphate, an important step in the biosynthesis of aromatic amino acids in plants.[10,11] Because glyphosate is the only herbicide that inhibits EPSPS, shikimate accumulation is considered an unequivocal indication of the effect of glyphosate on susceptible plants.[12] Species of the genus *Chloris* are distributed worldwide. They have slow initial growth, mainly in shady conditions or at low temperatures, but can be found in semiarid areas.[13] In Mexico, *Chloris* species are invasive weeds found in both altered and conserved areas.[14] *Chloris barbata* (L.) Sw. is widely distributed in the coastal states of Mexico,[14] coinciding with the main citrus-producing regions.

It is worth noting that species of the genus *Chloris* have a certain level of natural tolerance to glyphosate in comparison with species of other genera,[15–18] which allows these species to survive and reproduce after field herbicide applications lethal to wild plants of other species.[19] The survival of weed individuals after repeated application of the same herbicide or action mechanism is due to evolutionary adaptations resulting in herbicide resistance.[20] Total or partial glyphosate resistance/tolerance may be due to

alterations in the target-site represented by single or double mutations in the conserved region of the *EPSPS* gene,[3,21] *EPSPS* gene duplication[22] or both.[4,23] Reduced uptake and/or impaired translocation and degradation of glyphosate into non-toxic substances as well as hypersensitive reactions have also been reported as non-target site resistance mechanisms contributing to the resistance to this herbicide.[24–26]

Chloris barbata individuals survived the widely used standard dose of 720g acid equivalent (ae) ha^{-1} of glyphosate in citrus-production systems from Colima. Loss of susceptibility to glyphosate in *C. barbata* may be due to an increase in its innate tolerance or the evolution of resistance mechanisms. The aims of this study were to characterize the different levels of glyphosate susceptibility in two putative resistant *C. barbata* populations, collected in Persian lime orchards from Colima, and the mechanism (s) governing their resistance/tolerance to this herbicide.

MATERIAL AND METHODS

Biological material and experimental conditions

Seeds from resistant *C. barbata* populations (R1 and R2), collected from at least 20 plants that survived the final glyphosate treatment of 720 g ae ha^{-1} in 2014, were harvested in two Persian lime orchards of the Valenzuela farm (18◦54′52″N, 103◦51′37″W), in the municipality of Tecoman, Colima.

Fields from which seed samples were taken were at least 600 m apart, and had a 7-year history of glyphosate application (three or four times per year). Glyphosate was always applied when weeds were well grown or setting seeds in these orchards. Weed management was also complemented with hand mowing three or four times per year 2–3 weeks prior to herbicide treatment. Seeds of a non-treated population (referred as S) were collected near the Persian lime groves (18◦59′38″N, 103◦50′46″W).

Seeds were sown in trays (15 × 15 × 8 cm) with peat substrate moistened to field conditions and covered with parafilm. The trays were taken to a growth chamber calibrated for 26/18 ^{o}C day/night, 16 h photoperiod at 850 μmol^{-2} s^{-1} of light intensity, and 60% relative humidity (RH). The seedlings were transplanted into 3 L pots (five plants per pot) containing a sand/peat mixture (1:1 v/v), before placing them back in the growth chamber. They were watered daily until glyphosate treatment.

A screening test was conducted on the R populations to eliminate susceptible individuals from the field-collected seed. Fifteen pots were treated with 720 g ae ha^{-1} of glyphosate (Roundup Energy 45% w/v, Monsanto, Madrid, Spain) using a Generation III Research Track Sprayer (De Vries Manufacturing Inc., Hollandale, MN, USA) chamber equipped with an 8002 EVS nozzle (TeeJet, Spraying System Spain, S.L., Madrid, Spain) calibrated to deliver 200 L ha^{-1} at 200 kPa at a height of 50 cm. Surviving individuals were grown to maturity, bulked and allowed to produce seeds.The new purified seeds were germinated under the conditions described above, but seedlings were transplanted individually into 250 mL pots. *Chloris barbata* plants with three or four true leaves were used for all experiments.

Dose–response

Plants from the three (S, R1 and R2) *C. barbata* populations were sprayed with the following increasing doses of glyphosate: 0, 62.5, 125, 250, 500, 1000, 2000, 3000 and 4000 g ae ha^{-1} in a treatment chamber. Three weeks after application, survival was assessed and the fresh shoots of the aerial part of plants were harvested and weighed. The experimental design included 10 replicates per dose and was repeated twice. Data were expressed in percentages.

Shikimic accumulation assay

Samples of 50 mg of young leaf tissue (4 mm leaf discs) were taken. Shikimic acid accumulation was determined according to Shaner *et al.*[27] The glyphosate concentrations used were: 0, 100, 500 and 1000 μM. Sample absorbance was measured using spectrophotometer (Beckman DU-640, Beckman Instruments Inc., Fullerton, CA, USA) at 380 nm. The experiments had a completely random design using three tissue samples from each *C. barbata* population per glyphosate concentration and they were repeated twice. Results were expressed in mg of shikimic acid per g of fresh tissue.

Uptake and translocation

Chloris barbata plants of the S, R1 and R2 populations were treated with a ^{14}C-glyphosate [glycine-2-^{14}C] (specific activity 273.8 MBq $mmol^{-1}$, American Radiolabeled Chemicals, Inc., St. Louis, MO, USA) + commercial glyphosate solution. The final glyphosate concentration corresponded to 360 g ae ha^{-1} in 200 L ha^{-1} which contained a specific activity of 50 000 dpm μL^{-1} (equivalent to 0.834 kBq μL^{-1}). Twenty

plants per population were treated with one drop (1 μL plant^{-1}) of solution on the adaxial surface of the first or second leaf. The plants were handled according to Alcántara-de la Cruz *et al.*[5] at 24, 48, 72 and 96 h after treatment (HAT) (five plants per population at each time evaluated in a completely random design). Radioactivity of ^{14}C was analyzed by liquid scintillation spectrometry in a scintillation counter (Beckman LS-6500, Beckman Coulter Inc.) for 10 min per sample. Radioactive values in dpm were used to calculate the percentages of ^{14}C-glyphosate recovered, taken up, and translocated.

Metabolism

Five plants from each *C. barbata* population were treated with 360 g ae ha^{-1} of glyphosate. Untreated plants were used as controls. Leaf tissues were washed with distilled water at 4 days after treatment, flash-frozen in liquid nitrogen, and stored at -40 ◦C until use. The methodology described by Rojano-Delgado *et al.*[28] was used to determinate the percentage of glyphosate and its metabolites (aminomethyl phosphonate (AMPA), glyoxylate, sarcosine and formaldehyde) by reversed polarity capillary electrophoresis using a 3D Capillary Electrophoresis Agilent G1600A instrument equipped with a diode array detector (DAD; wavelength range 190–600 nm). Standard compounds used were provided by Sigma-Aldrich (Madrid, Spain). Glyoxylate naturally produced (untreated plants) was subtracted from the average of glyoxylate produced from glyphosate metabolism (treated plants) for each biotype. The experiment had a completely randomized design and was repeated twice. Data were expressed as percentages fromthetotal of glyphosate plus metabolitesthatwere recovered.

EPSPS enzyme activity

One 5 g sample of young tissue was collected from 20–30 plants from each *C. barbata* population. Samples were ground to a fine powder in liquid nitrogen in a chilled mortar, and enzyme extraction was performed following the protocol described by Sammons *et al.*[29] The total soluble protein (TSP) in the extract (basal activity in absence of glyphosate) was measured using a Kit for Protein Determination (Sigma-Aldrich) following the manufacturer's instructions. Specific EPSPS activity was assayed in the presence of glyphosate (0, 0.1, 1, 10 100 and 1000 μM) using the EnzChek Phosphate Assay Kit (Invitrogen, Carlsbad, CA, USA). EPSPS activity was measured for 10 min at 360 nm in a spectrophotometer (Beckman DU-640) to determine the amount of

inorganic phosphate (μmol) released, as measured in μg^{-1} TSP min^{-1}, and expressed as a percentage relative to the control (absence of glyphosate). The experiment was repeated twice with three technical replications at each glyphosate concentration.

EPSPS gene sequencing

Total RNA was extracted from 10 plants from each *C. barbata* population following the methodology described by Pistón.[30] RNA integrity was verified in 0.8% agarose gel and quantified in a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). First-strand complementary DNA (cDNA) synthesis was carried out with 1 μg of RNA per sample using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The degenerate primers BpF13(5′-TTGCCYGGRTCMAAGTCTTT-3′) and BpR11 (5′-TCCCAASTATCACTRTGTTC-3′), designed from *EPSPS* gene sequences of different weed species,3 were used to amplify a 639 bp fragment. The polymerase chain reaction (PCR) conditions described by Alcántara-de la Cruz[3] were followed in a total volume of 25 μL per reaction [50 ng of cDNA, 0.2 μM of each primer, 0.2 mM dNTP mix (PE Applied Biosystems; Life Technologies S.A., Spain), 2 mM $MgCl_2$, 1× buffer and 0.625 units of a 100: 1 enzyme mixture of non-proofreading (*Thermus thermophilus*) and proofreading (*Pyrococcus furiosus*) polymerases (BIOTOOLS, Madrid, Spain)]. Each PCR was carried out in triplicate. Ten microliters of PCR product was checked by 1% agarose gel, and 15 μL were reserved for cloning. Amplicons were cloned into competent cells of Escherichia coli (DH5α) and positive transformants were confirmed through PCR using the universal primers M13F (5′-GCCAGGGTTTTCCCAGTCACGAC-3′) and M13R (5′-TCACACAGGAAACAGCTATGAC-3′).[3] The plasmids were purified using the ilustra plasmidPrep Mini Spin kit (GE Healthcare, Little Chalfont, UK), and Sanger sequencing of two or three clones per plant was performed by STAB VIDA (Caparica, Portugal). Sequence assembly was carried out using the SeqMan Pro 11.0 (DNASTAR, USA) and Geneious 8.1.8 (Biomatters Ltd., New Zealand) software programs.

Statistical analysis

Data percentages for fresh weight reduction, survival and EPSPS enzyme activity were submitted to a non-linear regression analysis to find out the amount of glyphosate needed to reduce the fresh weight (GR_{50}), cause mortality (LD_{50}), and inhibit EPSPS activity (I_{50}) by 50% of each *C. barbata* population. The log-logistic equation used is: *Y*

$= c + \{(d - c)/[1 + (x/g)b]\}$,[31] where Y is the percentage of fresh weight, mortality and/or EPSPS enzyme inhibited relative to the control; c and d are the lower and upper limits, respectively, of the curve; b is the slope at the inflection point (i.e., GR_{50}, LD_{50} or I_{50}); and x is the glyphosate dose. Regression analyses were conducted using the *drc* package with program R version 3.2.5. Data of shikimic acid, basal EPSPS activity, uptake, translocation and metabolism were subjected to analysis of variance (ANOVA). Model assumptions of normal distribution of errors and homogeneous variance were graphically inspected. Differences with $P<0.05$ were considered significant and Tukey's test was conducted for means comparison.

RESULTS

Dose–response

Different glyphosate susceptibility levels were corroborated between S and R *C. barbata* populations. The LD_{50} for the S population was 544.9 g ae ha^{-1}, whereas the values for the R1 and R2 populations were 2295.6 and 3127 g ae ha^{-1}, respectively.

At 1000 g ae ha^{-1}, < 20% of S plants survived, and at 2000 g ae ha^{-1}, all plants of this population died. The fresh weight reduction (GR50) of S population was 4.8 and 6.4 times larger in relation to the R1 and R2 populations, respectively (Table 1, Fig. 1).

Table 1. Glyphosate dose/concentration required to kill individuals of a population (LD_{50}), reduce the fresh weight (GR_{50}) and/or inhibit the EPSPS activity (I_{50}) by 50% in glyphosate-resistant (R1 and R2) and -susceptible (S) *Chloris barbata* populations

Population	GR_{50} (g ae ha^{-1})	RI	LD_{50} (g ae ha^{-1})	RI	I_{50} (μM)	RI
R1	613.9 ± 92.8	4.8	2295.6 ± 391.9	4.2	112.9 ± 19.5	12.0
R2	810.4 ± 136.1	6.4	3127.5 ± 476.6	5.7	138.3 ± 26.7	14.7
S	127.1 ± 4.1	-	544.9 ± 22.4	-	9.4 ± 0.9	-

RI, Resistance indexes (R/S) calculated using LD_{50}, GR_{50} or I_{50} of the respective resistant population and LD_{50}, GR_{50} or I_{50} of the susceptible population. Values are given ±95% CI.
[a] $n = 10$ per glyphosate dose.
[b] $n = 3$ technical replicates per glyphosate concentration.

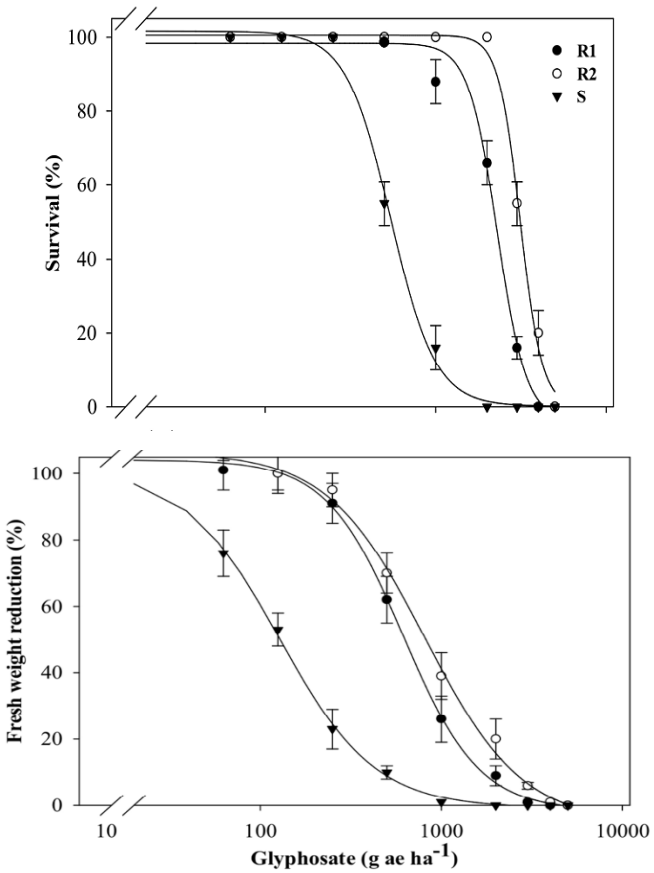

Figure 1. Dose–response curves of survival (upper) and fresh weight reduction (lower) in glyphosate-susceptible and -resistant Chloris barbata. Vertical bars represent the standard error of the mean (n = 10 per glyphosate dose)

Shikimic acid accumulation

The amount of shikimic acid in the absence of glyphosate was similar between the R nd S populations. Once treated, the three *C. barbata* populations accumulated shikimic acid as the glyphosate doses gradually increased. The accumulation was markedly higher in the S plants than the R plants. The latter (R1 and R2) presented similar patterns of shikimic acid accumulation. At 1000 μM glyphosate, S plants accumulated between 4.3 and 5.2 times more shikimic acid (~ 225 mg shikimic acid g^{-1} fresh weight) than R1 and R2 plants, respectively (Fig. 2).

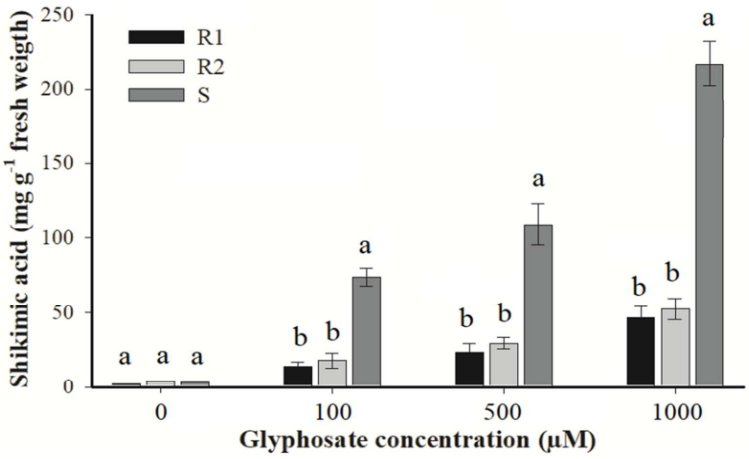

Figure 2. Shikimic acid accumulation of glyphosate-susceptible and -resistant Chloris barbata plants at different glyphosate concentrations. Groups of bars with the same letter above them are not different using the Tukey test at 95%. Vertical bars represent the standard error of the mean (n = 3 per glyphosate concentration).

Uptake and translocation

The uptake of 14C-glyphosate ranged from 15.3 to 20.9% at 24 HAT, and from 30.4 to 32.9% at 96 HAT. There were no significant differences in ^{14}C-glyphosate uptake between R and S C. barbata populations (Fig. 3); however, there were differences in the ^{14}C-glyphosate translocation. S plants moved at least 12% more herbicide to the rest of plant and root system at 96 HAT, compared with R plants. Therefore, we found ~ 4–7% more radiolabeled herbicide in the rest of plant, and up to 5–9% in the roots of S plants (Table 2).

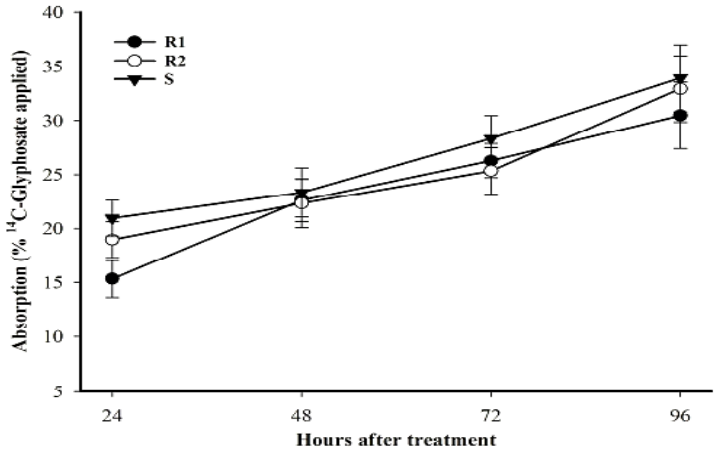

Figure 3. 14C-Glyphosate uptake in glyphosate-susceptible and –resistant Chloris barbata plants 24–96 h after treatment. Vertical bars represent the standard error of the mean (n = 5 per time evaluated).

Glyphosate metabolism

The metabolism study showed no significant differences in the quantified amounts of 210 glyphosate and its metabolites, except for AMPA, between the R and S *C. barbata* populations. At 96 HAT, ≈95% of glyphosate was not metabolized by the plants. The levels of AMPA and glyoxylate ranged from 2.8 to 4.3% and 0.8 to 1.3%, respectively; meanwhile sarcosine was not detected (Table 2).

Table 2. Percentages of translocation and metabolism of ^{14}C-glyphosate in resistant (R1 and R2) and susceptible (S) *Chloris barbata* populations at 96 h after treatment

	Translocation[a] (from % absorbed)			
Population	Treated leaf	Rest of plant	Root system	
R1	67.9 ± 4.1 a	18.8 ± 1.8 b	13.3 ± 2.6 b	
R2	64.2 ± 3.9 a	21.7 ± 2.3 ab	15.1 ± 2.4 b	
S	53.4 ± 3.7 b	25.4 ± 3.3 a	21.2 ± 2.1 a	
	Metabolites (%)			
	Glyphosate	AMPA	Glyoxilate	Sarcosine
R1	94.6 ± 1.4	4.3 ± 0.7 a	1.1 ± 0.3	ND
R2	96.4 ± 1.7	2.8 ± 0.5 b	0.8 ± 0.3	ND
S	95.3 ± 1.5	3.4 ± 0.5 ab	1.3 ± 0.5	ND

[a] Percent of herbicide-labeled absorbed. Entries within a column followed by the same letter are not statistically different using the Tukey test at 95%. Values are given ± the standard error of the mean ($n = 5$). AMPA, aminomethylphosphonic acid.

EPSPS enzyme activity

The amount of glyphosate needed to inhibit the EPSPS activity by 50% (I_{50}) in the S population was 9.4 µM. Regarding this value, the R1 and R2 populations were 11.0 and 14.7 times more resistant, respectively, than the S population (Table 1, Fig. 4). Meanwhile the specific activity of EPSPS in absence of herbicide showed no differences between the R and S *C. barbata* populations, with averages of 0.033 ± 0.005 (R1), 0.036 ± 0.080 (R2) and 0.032 ± 0.008 (S) µmol Pi μg^{-1} TSP min^{-1}.

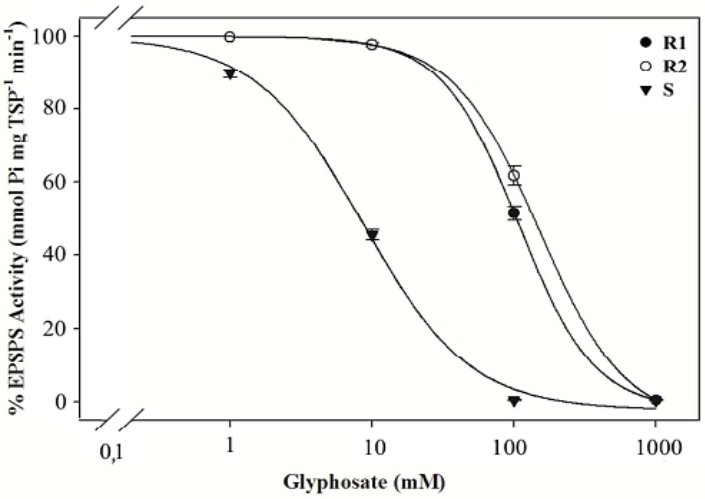

Figure 4. EPSPS enzyme activity expressed as percentage of the untreated control in leaf extracts of glyphosate-susceptible and -resistant *Chloris barbata* plants. Vertical bars represent the standard error of the mean (n = 3 per glyphosate concentration).

Sequencing of the EPSPS gene

The sequences of the EPSPS genes from *C. barbata* populations were aligned and numbered based on the known EPSPS sequence of *Leptochloa virgata*. Different single nucleotide polymorphisms (SNPs) between species were observed, but with a homology >97% at the protein level. The R populations of *C. elata* showed an amino acid substitution (R1 = 8 and R2 = 7) at the 106 position consisting of a proline (CCG) to serine (TCG) (Fig. 5). Of the 10 individuals sequenced per R population, eight and seven plants of the R1 and R2, respectively, presented the mutation Pro106-Ser, i.e., 80% and 70% of the sample size analyzed.

Amino acid position				100		102				106				110	
L. virgata S	TTG	GGG	AAC	GCT	GGA	ACT	GCG	ATG	CGG	CCA	TTG	ACG	GCT	GCT	GTA
(KX425854)	L	G	N	A	G	T	A	M	R	P	L	T	A	A	V
Consensus of	CTT	GTT	AAT	GCA	GGA	ACC	GCT	ATG	CGC	CCG	TTG	ACC	GCT	GCA	GTT
C. barbata	L	G	N	A	G	T	A	M	R	P	L	T	A	A	V
R1	L	G	N	A	G	**T**	A	M	R	**S**	L	T	A	A	V
R2	L	G	N	A	G	**T**	A	M	R	**S**	L	T	A	A	V
S	L	G	N	A	G	**T**	A	M	R	**P**	L	T	A	A	V

Figure 5. Partial alignment of nucleotides and amino acid sequences of EPSPS genes of glyphosate-susceptible and -resistant *Chloris barbata* populations.The yellow color indicates nucleotide changes among species. The green color indicates an amino acid change at the 106 position from proline (CCG) to serine (TCG). Box includes positions 102 and 106 corresponding to mutations confirmed to confer glyphosate resistance.

DISCUSSION

The resistance to glyphosate of the R1 and R2 *C. barbata* populations was confirmed, because their GR_{50} values were close to the standard dose of 720 g ae ha^{-1}, and the LD_{50} values were above this value (Table 1). In addition, the S *C. barbata* population showed LD_{50} value (545 g ae ha^{-1}) close to the standard dose, evidencing a certain innate tolerance level in this species. Sensitive populations of *C. elata*, *C. ciliata*, *C. truncata* and *C. virgata* also recorded high degrees of tolerance to glyphosate with LD_{50} ranging from 515 to 1385 g ae ha^{-1} of glyphosate.[16–18] Taking into account that controlling a weed population requires at least twice its estimated LD_{50}, [3] the standard dose of 720 g ae ha^{-1} used is not enough to control the S *C. barbata* population (Fig. 1). Depending on the cropping system, weed species, and infestation level, the effective glyphosate doses may range from 700 to 2 100 g ae ha^{-1}.[32] It would be more appropriate to apply doses >1100 g ae ha^{-1} (twice the LD_{50} of population S) to achieve an acceptable control of *C. barbata*, but the Persian lime farmers from Mexico have widely adopted the standard dose of 720 g ae ha^{-1}, because this dose controls a large number of weeds and they often have to make several glyphosate applications per year. However, this glyphosate dose results in low levels of control for *C. barbata*. Unfortunately, low herbicide doses can select for resistant phenotypes quicker than the recommended dose.[33] As a consequence, the R1 and R2 *C.barbata* populations increased their tolerance level and possibly developed resistance mechanisms against glyphosate. Therefore, alternative herbicides, such as diuron + paraquat, glufosinate, and glufosinate + indaziflam, that showed satisfactory control in glyphosate-resistant *Leptochloa virgata* populations in Persian lime orchards from Veracruz, Mexico,[8] may be applied to achieve an acceptable control of *C. barbata*. The loss of susceptibility to glyphosate in the R *C. barbata* plants was reflected in their low accumulation of shikimic acid compared with S plants (Fig. 2). The lower shikimate accumulation in R plants indicates limited interaction of glyphosate with the EPSPS protein, and may be due to either target site or non-target site resistance mechanisms.[34] However, it is difficult to deduce the mechanism (s) involved in the resistance of each resistant *C. barbata* population based solely on shikimate accumulation, because this parameter is just a resistance indicator.[4] ^{14}C-glyphosate uptake was similar between R and S *C. barbata* plants (Fig. 3), observations that are in agreement with the results reported for *C. virgata* plants from Australia, which did not present differences in their uptake patterns.[18] However, the S *C. barbata*

plants translocated slightly more ^{14}C-glyphosate than the R plants (Table 2). Any reduction in translocation negatively affects glyphosate efficiency.[35] This is a mechanism that is not completely understood, but an unknown barrier in the phloem system or in the mesophyll cells has been suggested,[10] altering the subcellular glyphosate distribution. In addition, it is suspected that potential ABC transporters could be involved in the impaired ^{14}C-glyphosate translocation.[36,37] These results suggest that translocation might be contributing to glyphosate resistance in these R *C. barbata* populations. However, it is difficult to determine the degree of their contribution to resistance, because the translocation differences were lower than those observed in R *C. elata* populations from Brazil,[38] where this mechanism played an important role. It is likely that resistance to glyphosate in *C. barbata* via reduced translocation is an evolving mechanism, because sublethal doses select genes related to non-target site mechanisms,[33] which could play an important role if steps are not taken to delay/stop the evolution of resistance.[20] The R and S *C. barbata* plants showed similar glyphosate metabolism rates (Table 2).

Glyphosate metabolism has been studied in various glyphosate-resistant and -tolerant weed species such as: *Colognia broussonetti*,[19] *C. elata*,[16] *Echinochloa colona*,[39] *Ipomea lacunosa*[40,41] and *Lolium spp*,[42] among others. However, the contribution of glyphosate metabolism as a resistance mechanism is not clear, and so far occurs at significantly lower rates in glyphosate than glyphosate-resistant transgenic crops carrying the glyphosate oxidoreductase (GOX) gene.[43] In this respect, these studies demonstrated that treated weed plants, be they susceptible, tolerant or resistant, can metabolize minimal amounts of glyphosate into non-toxic substances, which could be a biological characteristic.[16] However, metabolism does not appear to be related to resistance to glyphosate of the R1 and R2 C. barbata populations.

Unlike the shikimate accumulation test, differences in the enzymatic activity of EPSPS directly evidence alterations in the gene encoding the target protein.[34] The similar basal activity between R and S *C. barbata* plants suggested that there was no EPSPS gene amplification in the R populations, although this mechanism was characterized as the major one that conferred resistance to glyphosate in R *C. truncata* plants from Australia.[18] In the absence of differences in the EPSPS basal activity, the higher I50 values in R *C. barbata* plants (Fig. 4) reveal mutations in the EPSPS encoding gene.

The EPSPS gene sequences of the R1 and R2 *C. barbata* populations showed the mutation Pro106-Ser (Fig. 5). Several mutations in the EPSPS gene have been

suggested as contributing to glyphosate resistance such as: Val133-Ile and Pro382-Leu in *E. indica*,[43] Asp71-Met, Ala112-Ile and Val201-Met in *Ophiopogon japonicus*, *Liriope platyphylla* and *L. spicata*,*44* and Glu91-Ala in *C. truncata*,[18] among others. However, the mutations responsable for conferring resistance to glyphosate must occur in the conserved region of the EPSPS gene, which includes amino acid positions 95 to 107,[4] as demonstrated in *E. coli*.[45,46] To date, only two mutations (Thr102 and Pro106) occurring in this region have been found.[3,21] Our results clearly demonstrate that the mutation Pro106-Ser was involved in the evolution of resistance to glyphosate in *C. barbata* R plants. The resistance levels associated with mutations in this position (from Pro to Ser, Ala, Leu or Thr) are well established in weeds, being two to three times higher tan the recommended doses. The mutations Pro106-Ser and -Leu/-Ser were identified in glyphosate-resistant populations of *C. elata* from Cuba[16] and *C. virgata* from Australia,[17] respectively, contributing totally or partially to their resistance.

CONCLUSIONS

The relatively 'low doses' of glyphosate (~ 720 g ae ha^{-1}) to control weeds in Persian lime orchards from Colima increased tolerance and selected for resistance to this herbicide in *C. barbata*. The amino acid change from Pro to Ser in the 106 position of the EPSPS gene governs the resistance to glyphosate of the R1 and R2 populations. The impaired translocation, an evolving non-target-site resistance mechanism which is being selected by the 'low glyphosate doses', could play an important role in the resistance. Therefore, adequate doses of glyphosate and alternative herbicides must be used for the management of *C. barbata*, which contribute to delaying/stopping the evolution of glyphosate resistance. These result confirm the first case of glyphosate resistance evolved in *C. barbata.*

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CAPÍTULO V

Evaluación de resistencia y estrategia de manejo químico de *Chloris virgata* Sw. y *Chloris elata* Desv.en Argentina

RESUMEN

El uso frecuente en el espacio y tiempo que se hace del glifosato en la región agrícola de Argentina produjo una severa presión de selección de malezas. Entre ellas, *Chloris virgata Sw.* y *Chloris elata* Desv. constituyen biotipos con amplia difusión en los campos agrícolas del centro y norte de Argentina. De acuerdo a lo citado los objetivos del trabajo fueron evaluar mediante curvas dosis-respuesta el nivel de mortalidad y resistencia al herbicida glifosato de las especies *C. elata* y *C.virgata* y evaluar la eficacia de control y costo de aplicación de herbicidas postemergentes y preemergentes alternativos y/o complementarios al glifosato. Los ensayos se realizaron durante los años 2015-2017 en condiciones semi controladas en laboratorio de Ecotoxicología e invernadero de FCA-UNC. Los tratamientos postemergenes se realizaron con *C.elata* en 4-5 hojas y con *C.virgata* en pre y en postmacollaje de la maleza en invernadero. Con base en los resultados obtenidos es posible concluir que biotipos de *Chloris virgata* y *Chloris elata* colectados en Argentina presentan resistencia con un FR de 4.09 y 2.6, respectivamente. *Chloris virgata* presenta alta sobrevivencia a dosis comerciales en el estado fenológico de 3-4 hojas. Para lograr un control de 95% con glifosato en macollaje representa un incremento de casi 33 % en el costo de control en relación a aplicaciones en premacollaje.Es sensible al herbicida haloxifop-p-metil con dosis igual o superior a 13.5 g.i.a.ha^{-1} en el estado fenológico de 3-4 hojas. En estado de macollaje se obtienen valores apropiados de control con la utilización de dosis comercial recomendada. *Chloris elata* es sensible a haloxifop-p-metil a partir de 54 g.i.a. ha^{-1} en 4-5 hojas. El control en preemergencia es deficiente con el herbicida Ligate (sulfometuron + clorimuron etil), los herbicidas imazetapir y diclosulam son igualmente eficaces en residualidad y cotrol sobre Chloris elata pero sin superar el valor de 72%. Los resultados obtenidos con *Chloris virgata* confirman el primer caso de resistencia de esta especie a glifosato en Argentina.

INTRODUCCIÓN

En Argentina, las malezas constituyen uno de los principales problemas de la agricultura (Molina, 2007, Novo *et al.,* 2016). En este país, después de mas de 20 años de siembra de soja transgénica, el continuo uso de glifosato en una superficie extensa de la región agrícola bajo siembra directa (mas del 70%), el desinterés por las rotaciones de cultivos, la escasa rotación de principios activos con diferentes modos de acción, la ocupación de tierras menos apta para la agricultura y el intenso desmonte, se están manifestando las hipótesis previas de reportes de investigaciones que predecían la aparición y difusión de especies de malezas difíciles de controlar, tolerantes y resistentes al glifosato (Bracamonte *et al.*, 2016; Rainero, 2008; Akobundu, 1989), entre ellas las del género *Chloris* (Altieri y Pengue, 2006).

El avance de las denominadas especies tolerantes y resistentes (Heap, 2017), entre ellas las del genero *Chloris*, no es una consecuencia directa del uso de glifosato, sino el uso sin criterio técnico que se hace de él en Argentina.

En Argentina existen al menos 15 especies del género *Chloris*, incluyendo a las del género *Trichloris* y otras especies pertenecientes a la tribu de las Chlorideas. Estas especies algunas son anuales, otras son perennes y no son homogéneas en cuanto su distribución geográfica así como en lo referente a su respuesta a herbicidas (Correll y Johnston, 1970; Cronquist *et al.*, 1994; Espinosa y Sarukhán, 1997; Gleason y Cronquist, 1991; McVaugh, 1983; Rzedowski y Rzedowski, 2004).

Las Chlorideas son especies con alta tolerancia al glifosato, en especial en estados avanzados de desarrollo (Bracamonte *et al.*, 2017a; Bracamonte *et al.*, 2017b; Barroso *et al.,*2014; Ngo *et al.,*2017). Debido a ello, los productores se ven necesitados de utilizar glifosato en mezclas con graminicidas postemergentes y con herbicidas residuales de suelo para disminuir la germinación del stand de semillas del suelo. Entre las especies que han incrementado en forma significativa su presencia en la región núcleo agrícola de Argentina (Figura 1) y particularmente en cultivos de soja y maíz de las regiones agrícola del centro de la provincia de Chaco, de La Pampa y norte de Córdoba, son las correspondientes al género *Chloris elata* Desv. *y Chloris virgata* Sw. (Aapresid-REM, 2018; Vitta *et al*.,1999; Leguizamón *et al.* 2006).

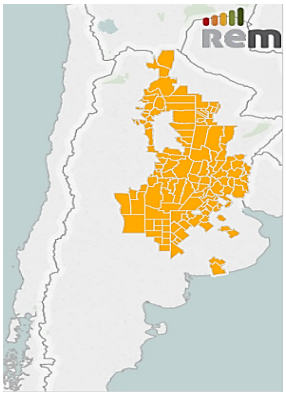


Figura 1. Área de cobertura de *Chloris spp* en la región productiva agrícola de Argentina. Aapresid-REM,2018.

C.elata (sinonimias:*C. arundinacea*, *C.consanguinea*, *C.dandyana,Chloris polydactyla),* de nombre común paja azul, es una especie de emergencia primavero-estival. La dinámica de rebrote y emergencia de *Chloris elata*, va desde agosto hasta mayo, teniendo su máximo crecimiento de febrero a marzo.

Es una planta perenne de 0.60-1.35 m de altura, erecta, cespitosa, raramente estolonífera. Poseen cañas delgadas, simples, excepcionalmente ramificadas, nudos glabros. Las vainas de las hojas alcanzan hasta de 17 cm long., se presentan glabras, escabrosas en los márgenes, pilosas hacia la zona ligular. La lígula es pilosa, con algunos pelos más rígidos hasta de 3-4 mm long. Las láminas son planas, hasta de 45 cm long. x 4-15 mm lat. La inflorescencia está constituida por 4-35 racimos espiciformes de 8-18 cm long., flexuosos o erectos, verticilados en el extremo de la caña. Poseen espiguillas unilaterales, imbricadas con 3-4- floras. Las glumas (2) son persistentes, lanceoladas, agudas, la inferior de 1-2.5 mm long., la superior de 1.9-3.5 mm long., de mayor longitud que los antecios. La lemma inferior es fértil de 1.5-2.6 (-2.8) mm long. x 0.5-1 mm lat., aquillada, carina ciliada, márgenes pilosos, pelos blanquecinos de 1.5-3 mm long., aristulada o con arista apical de (1-1.4-)1.7-1.8 mm long., recta u oblícua, escabrosa. Pálea de 1.5-2 mm long. x 0.7-0.8 mm lat., anchamente elíptica, dorso piloso, carinas ciliadas, ápice agudo. Las lodículas (2) están soldadas a la base de la pálea. El androceo (3), posee anteras entre 0.6-1 mm long. El antecio superior es estéril, de 1-1.6 mm long., es obtuso, con arístula subapical de 1.7-4 mm long. Los antecios superiores siguientes son reducidos, entre 0.5-0.9 mm de longitud. Los cariopsis poseen 1-1.5 mm long. x 0.6 mm lat., castaña, trígona, plano-convexa, surco no diferenciado; mácula embrional 1 /2 a 3 /4 de su longitud; hilo basal punctiforme (Molina & Rúgulo, 2004; Anderson, 1974) (Figura 2).

Figura 2. *Chloris elata*; a) planta macollada con inflorescencias, b) detalle de lígula, c) inflorescencia d) espiguilla. Foto a, b y c: nomalezas.com.ar, Foto d: plants.usda.gov.

La floración es despareja y prolongada, observándose dos picos, uno en primavera y otro en otoño (Fernández *et al*.,1993). Es una especie muy competitiva, generando en los lotes manchones de altos niveles de infestación, comprometiendo el normal desarrollo de los cultivos.

Esta especie está en constante expansión en las regiones agrícolas, observándose habitualmente en lugares sin disturbio alrededor de los campos de cultivos. Esta situación facilitó su adaptación a sistemas de siembra directa, invadiendo actualmente, importantes superficies cultivos de producción extensiva de soja y maíz de la región central de Chaco.

*C. virgata s*on plantas con cañas erectas, amacolladas, de 30 a 100 cm de altura. Hojas glabras, de lígulas cortas, ciliadas; láminas lineales planas y glabras de 80 a 150 mm de largo por 3-6 mm de ancho. Inflorescencia formada por 6 a 15 espigas erectas, de 3 a 10 cm de argo. Espiguillas de 3 a 3,5 mm de longitud. glumas lanceoladas mucronadas, la inferior mucho más corta que la superior. Antecio inferior fértil con 2 mechones de pelos largos en la arte superior de los márgenes, con arista de la misma longitud que su cuerpo. Antecio estéril, truncado en el ápice, aristado (Rúgolo de Agrasar *et al*., 2005) (Figura 3).

Figura 3. *Chloris virgata.* Planta completa, semilla e inflorescencia

Esta especie es la única que posee ciclo anual, por lo tanto posee mejor estrategia de competencia y perpetuación (mayor producción de semillas y germinación) respecto del resto de las chlorídeas perennes. Actualmente se ha constituido en uno de los principales problemas de malezas (Vitta *et al*.,1999; Leguizamón *et al.,* 2006) y ha comenzado a adquirir importancia en los sistemas agrícolas del área central y norte de Argentina (Figura 4). En campos con elevada infestación de esta especie forma rodales que no permite el establecimiento del cultivo, alcanzando pérdidas superiores al 80%.en diferentes cultivos estivales. En la región centro norte de Córdoba y en la región del noreste de La Pampa esta especie constituye una maleza de gran expansión y dificultad para su control. El patrón de emergencia en la región centro norte de Córdoba muestra un inicio y crecimiento rápido y sostenido desde inicio de primavera hasta inicio de verano, con varios flujos de emergencia en este periodo (Ustarroz, 2015). Esto valores son similares al reportado por Metzler *et al*. (2014) para un biotipo de Monge, Santa Fe.

Por ello, la identificación de las especies presentes, el conocimiento de su bioecología, su dinámica poblacional, nivel de infestación y la relación del herbicida y la sensibilidad de la mala hierba a nivel de campo e invernadero (Labrada, 1992) constituyen una base fundamental para un manejo de control eficiente. En este contexto, establecer la relación entre la dosis y la respuesta de las malezas a los herbicidas constituye la base para la comprensión de la eficacia del herbicida, su modo de acción y la especie considerada.

Esta metodología, ampliamente utilizada en el mundo como base para recomendaciones de dosis de uso comercial, evalúa la reacción de una maleza a distintas dosis de un mismo herbicida, solo o en mezcla, determinando valores de sensibilidad, tolerancia o resistencia en relación a un testigo sin control químico comprobable (Seefeldt *et al.,* 1995; Streibig *et al.*,1993).

El uso de regresión no lineal descrito por Streibig *et al.,*(1993), constituye el método más apropiado para el desarrollo de la curva de dosis-respuesta entre los herbicidas y de malezas. Una adaptación de este modelo y otros presentada originalmente en la literatura fue propuesto por Seefeldt *et al.,* (1995). Estos autores sugieren que el modelo log-logística tiene varias ventajas sobre otros métodos de análisis. La principal es que uno de los términos que integran la ecuación no lineal es la GR_{50} (llamada también ED_{50} o IC_{50}), facilitando de esta manera la comparación del nivel de resistencia de biotipos de la misma especie.

En estudios de tolerancia la respuesta binaria es el resultado clásico. Ejemplos típicos son los estudios en los que se denomina "Dosis-Respuesta," como el utilizado para determinar la dosis letal 50 (DL_{50}) de un herbicida. Si un individuo muere cuando es desafiado con una dosis $x > T$, se dice que el individuo tiene una tolerancia T. La tolerancia de las malezas varía entre individuos y puede considerarse una variable aleatoria y $F(t)=P(T<t)$. Si F(t) es la función de distribución normal estándar acumulada. El modelo apropiado para analizar estos ensayos es ajustando un modelo de regresión logística como el Probit (Balzarini *et al.*,2008).

De acuerdo a lo citado anteriormente es que se propone los siguientes objetivos:

Objetivo General

Evaluar el nivel de tolerancia o resistencia y mortalidad al herbicida glifosato de biotipos de *Chloris virgata y Chloris elata* de dos regiones agrícolas de Argentina y evaluar estrategias químicas alternativas o complementarias al glifosato.

Objetivos Específicos

A- Evaluar el nivel de resistencia y mortalidad al herbicida glifosato a campo y en condiciones semi controladas de un biotipo de *C. virgata y* un biotipo de *Chloris elata* de dos regiones agrícolas de Argentina.

B- Evaluar en forma prospectiva la eficacia de control y costo de aplicación de herbicidas preemergentes y postemergentes alternativos y/o complementarios al glifosato en el control de *Chloris virgata* y *Chloris elata*.

D- Diseñar y proponer estrategias de manejo integradas, complementarias y alternativas de *Chloris virgata* y *Chloris elata* en Argentina.

MATERIAL Y METODOS

El trabajo de investigación se inicio con un ensayo de prospección a campo en la zona rural de la ciudad de Rio Primero (31°15'27,88" S y 63°26'30,05" O), Córdoba (Figura 4 y Figura 5).

Figura 4. Region con alta difusión y dificultad de control de *C. virgata* en la zona rural de la ciudad de Rio Primero, Córdoba. (Figura 5).

Figura 5. Desarrollo del ensayo a campo en el control de *C. virgata*, Rio Primero, Cordoba, 2015.

Para evaluar el nivel de tolerancia del biotipo se seleccionaron dos herbicidas de diferente modo de acción, glifosato 66.2 % (equivalente ácido 54% p/p) y haloxifop-p-metil 54 % aplicados a tres diferentes y una dosis, respectivamente (Tabla 1).

Tabla 1. Tratamientos herbicidas en postemergencia de *C.virgata*. Rio Primero, Córdoba.

Momento	Nº Trat.	Ingrediente activo C.virgata	ia ha^{-1} (g/ cc)	ea ha^{-1} (g/ cc)
Post emergencia	1	Testigo	0	0
	2*	**Glifosato**	**1324**	**1080**
	3	Glifosato	2648	2160
	4	Glifosato	3972	2144
	5	**Haloxifop- p-metil**	**54**	-

Trat.2: Dosis recomendada comercialmente

Con el objetivo de evaluar la eficiencia de control y el nivel de tolerancia a herbicidas alternativos al glifosato, se utilizo herbicidas de los grupos químicos triazolpirimidinas, imidazolinonas y sulfonilureas aplicados en preemergencia de *C.virgata*. Los tratamientos herbicidas y las dosis seleccionadas son presentados en Tabla 2.

Tabla 2. Tratamientos herbicidas en preemergencia de *C.virgata*. Rio Primero. Cordoba.

Momento	**Trat. Nº**	**Ingrediente activo**	**iaha^{-1} (g o cc)**
Preemergencia	1	Diclosulam	25,2 g
	2	Imazetapir	106 g
	3	Sulfometuron + Clorimuron etil*	35 g
	4	Testigo	0

*En adelante Ligate (mezcla comercial recomendado para Soja STS, tolerantes a sulfonilureas).

Las aplicaciones se realizaron con una mochila de aire comprimido equipada con pastillas de abanico plano 110-015 en posemergencia cuando las malezas presentaban 4-5 hojas verdaderas y 110-02 paara tratamientos en prememergencia, que asperjaban un caudal de 100 L ha-^{1}. La cobertura vegetral del área de ensayo fue superior al 80%. Las parcelas (unidades experimentales) fueron de 2.5 metros de ancho por 8 metros de largo. Las condiciones ambientales en el momento de la aplicación en preemergencia y postemergencia fueron: T 27°C, HR 60 %, viento a 9 Km/h. Las evaluaciones de control visual (%) se realizaron a los 15 y 21 días después de las aplicaciones (DDA). Antes de los 15 días posteriores a la aplicación se realizo un riego de 15 mm con el objetivo de distribuir los herbicidas en el perfil superior del suelo.

Con el objetivo de contrastar los resultados obtenidos a nivel de campo se desarrolló un ensayo en condiciones semicontroladas (campo-invernadero) en el laboratorio de Ecotoxicología de la Facultad de Ciencias Agropecuarias de la Universidad Nacional de Córdoba durante el presente año (2015 y 2016). Semillas del biotipos de *C. virgata* con

alta dificultad de control se recolectaron en la zona del ensayo a campo de la ciudad de Rio Primero, Córdoba (Figura 7).

Para constrastar el nivel de susceptibilidad *de C. virgata* con una especie de *Chloris* perenne como *Chloris elata* en condiciones semicontrolada (invernadero-campo) se seleccionó y colectó semillas de un biotipo con dificultad de control con glifosato en la zona rural de la ciudad de Presidencia Roque Sáenz Peña, Chaco (en adelante biotipo Saenz Peña) en enero del 2016 (Figura 6).

Figura 6. Zona rural de Presidencia Roque Sáenz Peña, Chaco, con alta difusión de *Chloris elata*. 2016.

Con las poblaciones colectadas de ambas especies se procedió a germinar las semillas en bandejas en cámara de germinación con temperatura de 20/28 ºC noche/día, 16 h de fotoperiodo con 350 mol/m^2. Las plántulas obtenidas fueron repicadas definitivamente en macetas (unidades experimentales) quedando 4 plantas por unidad experimental. Posteriormente las macetas fueron mantenidas en condiciones controladas en invernadero para su posterior evaluación de control. En todos los tratamientos se realizaron riegos requerimiento para evitar stress hídrico. Para evaluar la sensibilidad de las malezas se realizaron aplicaciones en postemergencia (*C.virgata y C.elata*) y en preemergencia para *C.elata*. Los ensayos se realizaron en condiciones semi-controladas (campo-invernadero) en macetas (unidades experimentales) de 1 kg que contenían una mezcla de tierra negra y arena, en una proporción 1:1 para postemergencia y 1:2 para preemergencia.

Para el control en postemergencia de ambas malezas, se seleccionaron dos herbicidas de diferente modo de acción, glifosato 66.2 % (equivalente ácido 54% p/p) para *C. virgata* y glifosato 79% (equivalente ácido 72% p/p) para *C. elata,* y haloxifop-p-metil 54 % aplicados a seis dosis diferentes, tomando como central la dosis recomendada por la empresa (Tabla 3).

Tabla 3. Tratamientos herbicidas postemergentes para control de *C. virgata y C. elata*, 2015/2016 y 2016/2017.

			C.virgata		***C . elata***	
Momento	*Nº Trat.*	*Ingrediente activo*	*ia ha^{-1} (g/cc)*	*ea ha^{-1} (g/cc)*	*ia ha^{-1} (g/ cc)*	*ea ha^{-1} (g/ cc)*
	1	Testigo	0	0	0	0
	2	Glifosato	331	270	256,75	234
	3	Glifosato	662	540	513,5	468
	4	**Glifosato**	**1324**	**1080**	**1027**	**936**
	5	Glifosato	2648	2160	2054	1872
	6	Glifosato	5296	4320	4108	3744
Post emergencia	1	Testigo	0	-	0	-
	2	Haloxifop-p-metil	13,5	-	13.5	-
	3	Haloxifop-p-metil	27	-	27	-
	4	**Haloxifop-p-metil**	**54**	-	**54**	-
	5	Haloxifop- p-metil	108	-	108	-
	6	Haloxifop-p-metil	216	-	216	-

Las aplicaciones se realizaron con un pulverizador manual de aire comprimido equipada con pastillas de abanico plano 110-015, que asperjaban un caudal de 100 L ha^{-1}. Las aplicaciones en *C.virgata* se realizaron en dos estadios, uno en premacollaje, con 3-5 hojas verdaderas (aplicación temprana) equivalente a BBCH 13-14 (BBCH, 2001) y otro posterior con macollos en desarrollo (aplicación tardía), equivalente a BBCH 22-23, (BBCH, 2001; Figura 6). En *C.elata* se realizaron cuando las malezas presentaban 4-5 hojas verdaderas, equivalente a BBCH 13-14 (BBCH, 2001) Figura 8).

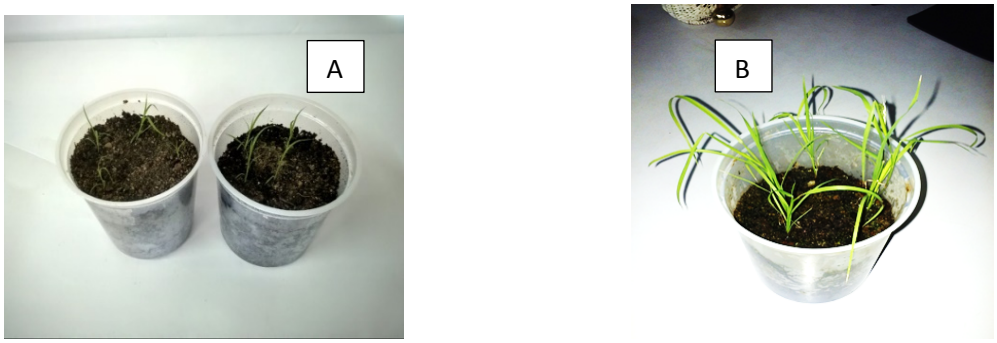


Figura 7. Estado fenológico del primer (A) y segundo momento (B) de control postemergencia de *Chloris virgata.*

Figura 8. Estado fenológico de control postemergente de *Chloris elata*.

Las condiciones ambientales en el momento de la aplicación en preemergencia y postemergencia fueron: T 24°C, HR 72%, viento de 5 Km/h. Las macetas fueron conservadas en condiciones controladas en invernadero y mantenidas con riego a discreción con el objetivo de distribuir el herbicida en el suelo (activación) y posibilitar que las semillas tengan suficiente humedad para germinar.

Las evaluaciones finales de control se realizaron a los 21 DDA. Se evaluó de manera visual el porcentaje de control y la Dosis Letal Media (DL_{50}) mediante el modelo de ajuste Probit, que representa la dosis (g.i.a. ha^{-1}) capaz de matar al 50 % de la población expuesta en relación a un control no tratado (testigo) y se evaluó GR_{50} (reducción de peso fresco (%) de las plantas tratadas con respecto a un control no tratado (testigo) para obtener la relación dosis-respuesta. Para ello se realizó un corte en la base de cada planta (al ras del suelo), y posteriormente fueron pesadas en balanza de precisión de 0.0001 g para determinar biomasa (gramo/planta) de peso húmedo.

Con los datos de peso obtenidos se procedió a evaluar la relación dosis-respuesta utilizando el modelo log-logístico propuesto por Seefeldt *et al.* (1995) que relaciona la respuesta de la planta con las dosis del herbicida:

$$\mathbf{Y} = \boldsymbol{C} + \frac{\boldsymbol{D} - \boldsymbol{C}}{\mathbf{1} + \mathbf{exp}(\mathbf{b}(\mathbf{log}(\mathbf{x}) - \mathbf{log}\,(\mathbf{GR50}))}$$

Donde:

Y: representa el peso fresco como porcentaje con respecto al control.

D: límite superior de la curva

C: límite inferior de la curva: respuesta media de la dosis más alta empleada.

b: pendiente de la curva en el punto GR_{50}.

GR_{50}: dosis correspondiente al 50% de reducción de crecimiento (peso)en relación al testigo.

Para determinar el ajuste del modelo se realizó a través del coeficiente de determinación R^2.

Para establecer el Factor de Resistencia relativa se estableció la siguiente relación: FR = $GR_{50}R/GR_{50}S$ donde:

$GR_{50}R$.=GR_{50} de *Chloris* sospechosa de resistencia y $GR_{50}S$= GR_{50} obtenida en ensayos paralelos con un biotipo *sensible* de *chloris virgata* ("General Pico", La Pampa).

El diseño experimental utilizado fue completamente aleatorizado, con 4 repeticiones por tratamiento y 5 testigos sin herbicidas.

En preemergencia, los herbicidas y dosis evaluados para *C.elata* fueron los mismos utilizados en ensayo a campo (Tabla 1). El diseño experimental utilizado fue completamente aleatorizado con 3 tratamientos herbicidas aplicados a la dosis comercial recomendada por la empresa y 5 repeticiones por tratamiento (Tabla 2).

Las evaluaciones de control se realizaron a 21 días después de aplicación (DDA), donde se determinó el número de plántulas emergidas y porcentaje de control en relación al testigo sin tratamiento herbicida. Los resultados obtenidos fueron evaluados mediante el sofware Insfostat (Di Rienzo *et al.* 2015). Para la significancia de los tratamientos se utilizó el ANAVA y para las diferencias significativas entre los tratamientos se utilizó el Test de LSD.

RESULTADOS Y DISCUSIÓN

Ensayo de Campo

Los resultados obtenidos en ensayo de campo mostraron baja suceptibilidad del biotipo Rio1 a glifosato en dosis iguales o superiores a la recomendada comercialmente, con valores de control apenas superando el 50%. Control satisfactorio, superando el 90 % se observo en tratamientos con haloxifop-p-metil con la dosis recomendada (Figura 9). Controles eficaces (>90%) se obtuvo con los herbicidas Ligate y diclosulam, pero con baja eficacia utilizando la dosis comercial de imazetapir (Figura 10)

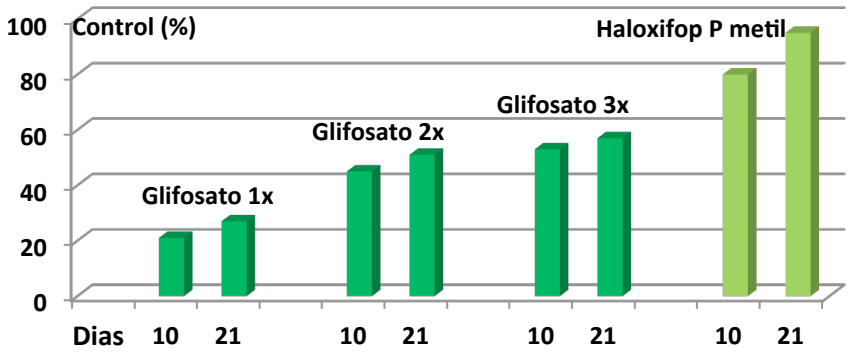


Figura 9. Control (%) de *C. virgata* con glifosato y haloxifop-p- metil. Rio Primero, Cordoba.

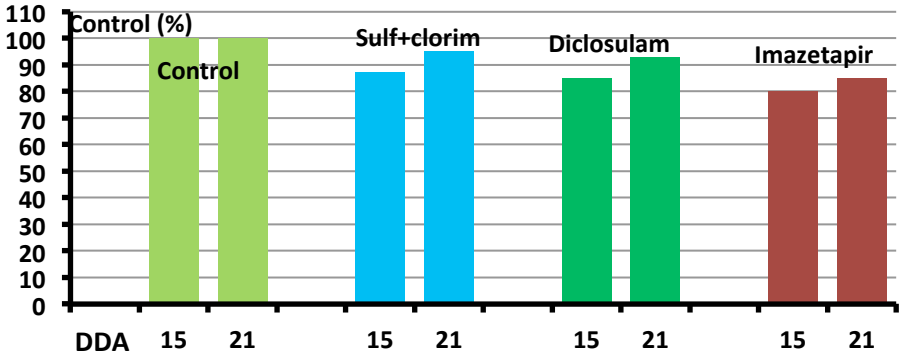


Figura 10. Control (%) de *C. virgata* con herbicidas residuales. Rio Primero, Cordoba.

Ensayos en Condiciones Semicontroladas

Eficacia de Control Sobre *Chloris virgata* de Herbicidas Postemergentes

Evaluación de tratamientos postemergentes en 3-5 hojas

La evaluación de control a los 21 DDA mostró que la eficacia de la dosis comercial en el estado fenológico recomendado por la empresa no fue el esperado, superando el 50% del control solo en la dosis 4x, alcanzando solo la D5 (5296 g.i.a./ha) un valor aceptable de control (87%) a nivel de campo (Figura 11).

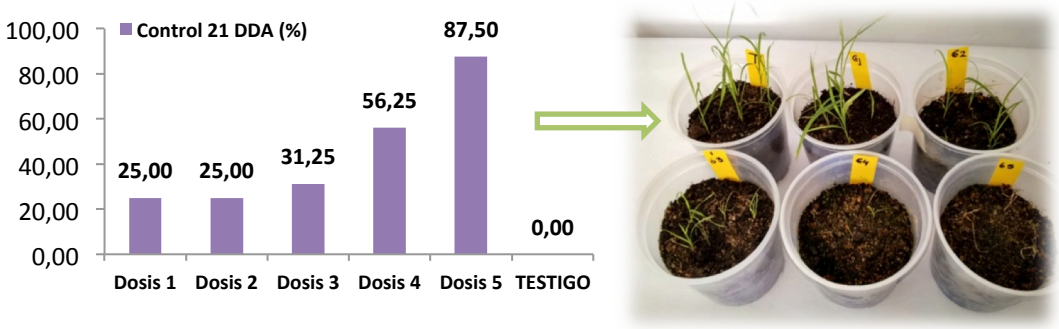


Figura 11. Control (%) de *Chloris virgata* con glifosato a los 21 DDA.

La baja eficacia observada en el control visual (%) es corroborado por la DL_{50} obtenida en la que se alcanzaron valores de 2.586 g.i.a. ha^{-1} de glifosato para el biotipo Rio1 (Figura 16) y 927 g.i.a. ha^{-1} para el biotipo General Pico. Estos resultados mostraron un índice de mortalidad (DL_{50}) de 2.78 (2586/927), evidenciando que se necesitan casi 3 veces más de ingrediente activo de glifosato para matar al 50% de la población del biotipo Rio Primero (A) en relación al biotipo sensible General Pico (B) (Figura 12).

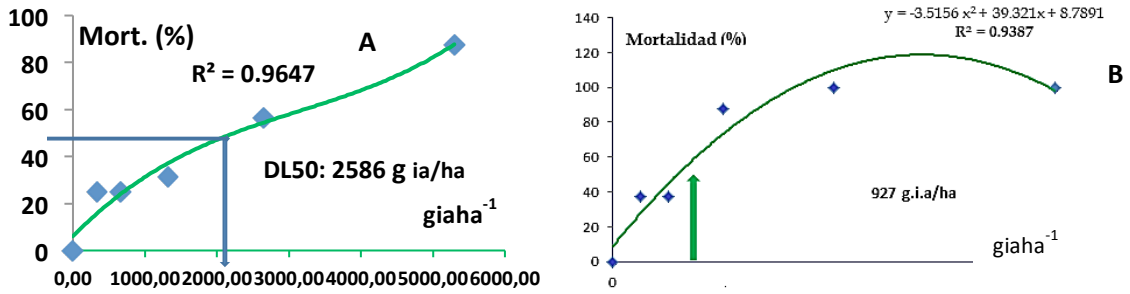


Figura 12. Dosis $Letal_{50}$ en el control de *Chloris virgata* con glifosato a los 21 DDA.

Los resultados observados en premacollaje a los 21 DDA mostraron una baja eficacia en la reducción del peso fresco de *Chloris* en todas las dosis evaluadas.

El ajuste de la relación dosis-respuesta mostró un valor de GR_{50} de 623 g.i.a. ha^{-1}, superior al obtenido en el biotipo General Pico. Este valor se localiza entre las dosis evaluadas D1 y D2. La GR_{50} obtenida en el biotipo Rio 1 resulto ***4.09 veces más resistente al glifosato en comparación con la población "General Pico (S).*** Estos valores indicarían que el biotipo Rio Primero se *presenta resistencia al glifosato* (FR > 2.5 (Tabla 4) Figura 13).

Tabla 4. Parámetros de la ecuación log-logística utilizada para calcular las dosis de glifosato requeridas para la reducción del 50 % del peso en fresco (GR_{50}) de las poblaciones de *Chloris virgata.*

Reducción de Peso Fresco (%)				
Población	***d***	***b***	**GR_{50} (g.a.i ha^{-1})**	**FR**
Río Primero	103.96	2.32	**623**	**623/152= 4.09**
General Pico	101.22	0.96	**152**	

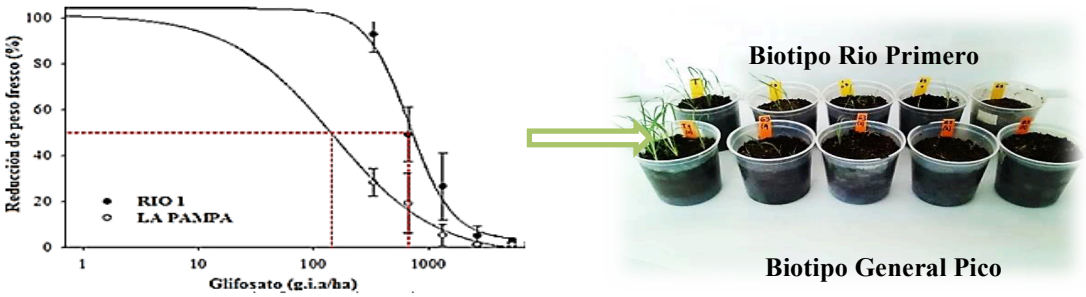


Figura 13 Curvas dosis-respuesta con glifosato sobre dos biotipos de *Chloris virgata* a 21 DDA.

Estos resultados también se tornan significativos en relación también a aquellos obtenidos por Ustarroz (2013) que evaluó los biotipos Manfredi (S) y Almafuerte que obtuvo valores de GR_{50} de 293.17 y 498.11, respectivamente. Los resultados también son coincidentes con lo relatado por Bracamonte *et al*, (2017) al describir al genero *Chloris* con tolerancia natural al glifosato. Tambien Metzler *et al.,*(2014), Barroso *et al.,*(2014) y Ngo *et al.,*(2017) sobre baja eficacia de control de *C.virgata* con glifosato en distintas poblaciones evaluadas.

Es importante destacar que las curvas dosis-respuesta de los biotipos (R y S) no son paralelas, lo que indicaria que la causa de la resistencia del biotipo R es debido a otros mecanismos de resistencia adicionales a la modificación del sitio de acción (Streibig *et al*.,1993, Bracamonte *et al*, 2017).

Con los resultados obtenidos, se puede observar que para alcanzar un control de 95% con glifosato se necesita 4913.4 g.i.a. ha^{-1} el cual genera un costo de 52.26 US$ ha^{-1} (Margenes Agropecuarios, 2016). Para obtener el mismo control con haloxifop-p-metil, se lo puede obtener con la mínima dosis evaluada (13,5 g.i.a. ha^{-1}), siendo necesario que las malezas a campo estén en estado 3-4 hojas. Los resultados también evidencian que con la utilización de las menores dosis de haloxifop-p-metil puede se logra el control total y seguro, con un costo mucho menor que glifosato, siendo de 6,5 US$ ha^{-1}. Estos resultados de control solo son posibles obtener a nivel de campo en base a un estricto plan de manejo del cultivo en general y de malezas en particular.

Evaluación en la eficacia de control de *Chloris virgata* con haloxifop-p-metil en 3-4 hojas.

Los resultados de control obtenidos a los 10 y 21 DDA con haloxifop-p-metil mostraron altos niveles de eficacia en todas las dosis evaluadas. A los 21 DDA con haloxifop fue posible observar 100% de control en todas las dosis evaluadas (Figura 14).

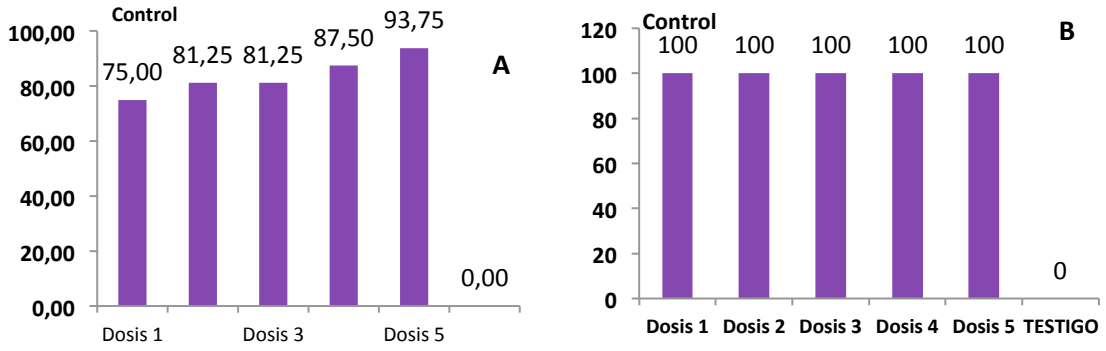


Figura 14. Porcentaje control de *Chloris virgata* con haloxifop a los 10 (A) y 21DDA (B)**.**

Estos resutados confirman los observados en ensayo de campo, donde en estadios tempranos (3–4 hojas) de *C. virgata* se obtienen controles rapidos (10 DDA) y totales (21DDA) en todas las dosis evaluadas. La eficacia obtenida con haloxifop son coincidentes con los obtenidos por Ustarroz (2015) y Metzler *et al.,*(2014) al obtener resultados satisfactorios a los 21DDA y en todas las dosis evaluadas. Los resultados obtenidos permiten pensar en alcanzar un buen control de *Chloris* con haloxifop a bajas dosis y con menores costos. Para alcanzar estos objetivos es necesario llevar un correcto plan de monitoreo de la maleza con el objetivo de dirigir la aplicación en su estado fenológico más susceptible y considerando el stand máximo de emergencia en el lote.

Evaluación de tratamientos postemergentes en macollaje de *Chloris virgata.*

En la etapa de macollaje se evidenció un aumento importante en la dificultad de control de *Chloris* con glifosato. A los 21 DDA la evaluación visual de control (mortalidad) mostro que solo la dosis 5 generó un mayor porcentaje de control (68.75%) pero aún lejos de un valor de control aceptable a nivel de campo (90-95 %) (Figura 15).

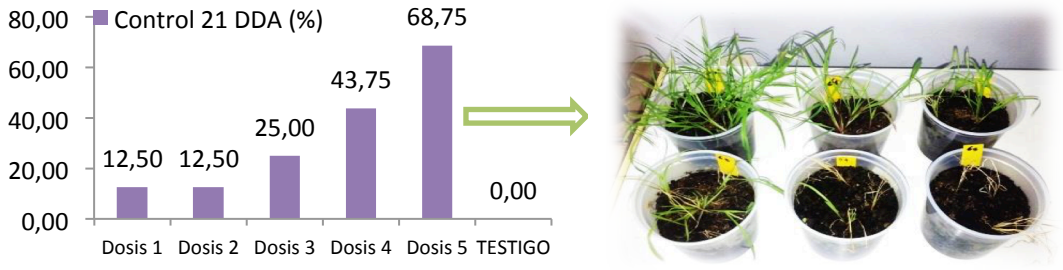


Figura 15. Porcentaje de control de *Chloris virgata* en macollaje con glifosato a los 21 DDA.

La baja eficacia en el porcentaje de control también fue observada en la DL_{50} obtenida alcanzando valores de 3595 g.i.a. ha^{-1} de glifosato para el control en macollaje (Figura 16) y 2586 g.i.a.ha^{-1} para el control en 3-4 hojas. Estos valores muestran un incremento de casi 40 % en la dosis de glifosato en aplicaciones en macollaje en relación a aplicaciones tempranas para alcanzar una mortalidad del 50% en relación a un testigo sin herbicida.

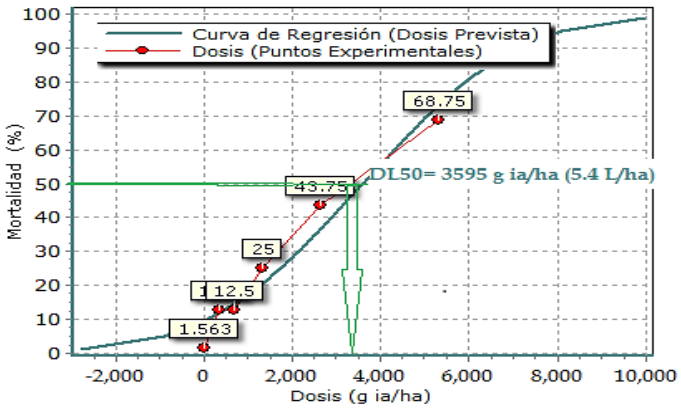


Figura 16. DL_{50} en el control de *Chloris virgata* en macollaje con glifosato a los 21 DDA.

Las diferencias en la reducción de peso a los 21 DDA entre las medias de los tratamientos mediante la prueba LSD mostro que solo la D5 un valor de casi 90% de reducción de peso fresco (Figura 17).

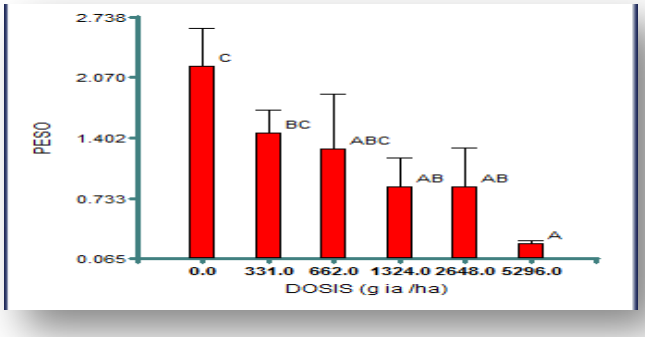
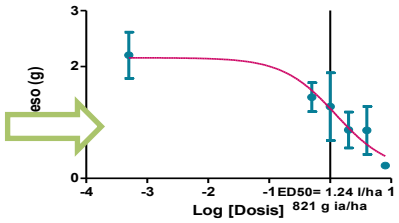


Figura 17. Medias y diferencias significativa entre dosis de glifosato mediante prueba LSD.

El ajuste de la relación dosis-respuesta mostró un valor de GR_{50} de 821 g.i.a. ha^{-1}, mostrando un aumento de más de 30% de dosis en relación a las utilizadas en aplicaciones realizadas en premacollaje lo que evidencia la mayor dificultad para su control (Tabla 5; Figura 18).

Tabla 5. Curva dosis respuesta y parámetros de la ecuación para calcular las dosis de glifosato requeridas para la reducción del 50 % del peso en fresco (GR_{50}) en dos estadios fenológicos de *Chloris virgata*.

Reducción de Peso Fresco (%)				
Control	***d***	***b***	**GR_{50} (g.a.i ha^{-1})**	**FR**
Premacollaje	103.96	2.32	623	**821/623= 1.31**
Macollaje	101.22	0.96	821	



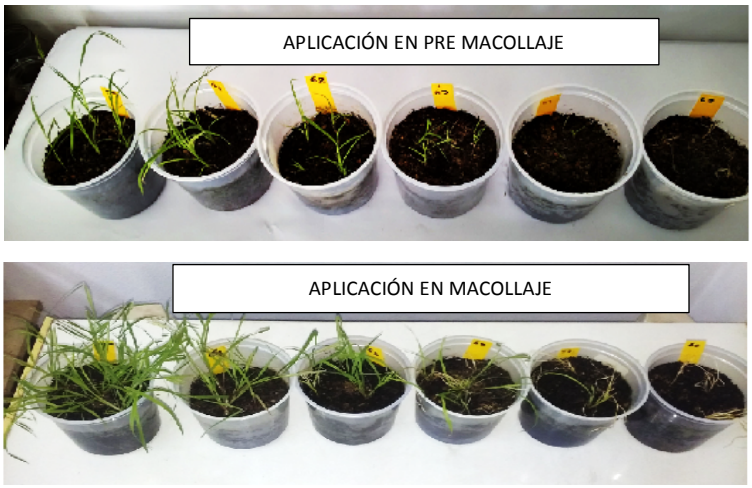


Figura 18. Dosis-respuesta con glifosato sobre *Chloris virgata* en distintos estadios fenológicos.

Evaluación en la eficacia de control de *Chloris* con haloxifop-p-metil en macollaje.

A los 21 DDA con haloxifop fue posible observar, ahún en estadio fenológico tardio, un 91.75% de control en la dosis recomendada comercialmente (Figura 19). Estos resultados muestran a haloxifop como una alternativa importante cuando se paso el momento optimo de control, permitiendo mantener prácticamente libre de competencia de *Chloris* hasta que cierre el cultivo de soja. Un adecuado control con este herbicida se puede alcanzar con dosis menores a la recomendada por el fabricante (54 g.i.a. ha^{-1}) donde se obtiene un control eficaz, con un costo menor en relación a glifosato (6,5 US$ ha^{-1}) (Margenes Agropecuarios, 2016). Estos valores de control solo son posibles obtener a nivel de campo en base a un estricto plan de manejo del cultivo en general y de malezas en particular.

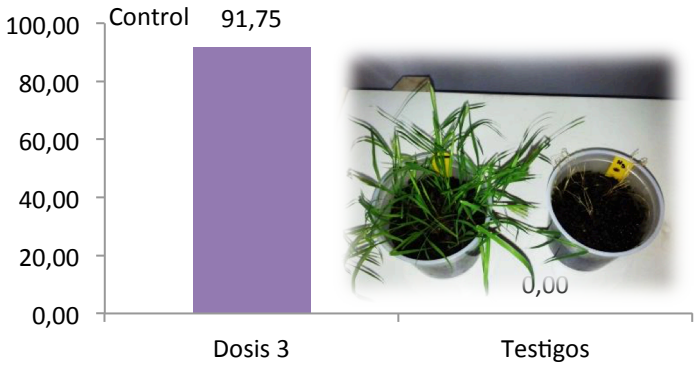


Figura 19. Porcentaje de control de *Chloris virgata* con haloxifop a los 21 DDA.

Eficacia de Control de Herbicidas Postemergentes sobre *Chloris Elata*

Evaluación en la eficacia de control de *Chloris elata* con glifosato

La evaluación dosis-respuesta mostro que el valor de GR_{50} del biotipo "Sáenz Peña" es **2,6** veces más tolerante que el biotipo sensible. Estos resultados evidencian que el biotipo "Sáenz Peña" se presenta como tolerante o con resistencia baja a glifosato (FR >2.5) (Tabla 6, Figura 20).

Tabla 6. Parámetros GR_{50} utilizada para calcular las dosis de glifosato requeridas para la reducción del 50 % del peso en fresco de las poblaciones de *Chloris elata T y S.*

Biotipo	GR 50 (g. e.i.a. ha^{-})	FR
Sáenz Peña	231(254 g.i.a. ha^{-1})	231/88= **2.6**

Sensible	**88**	

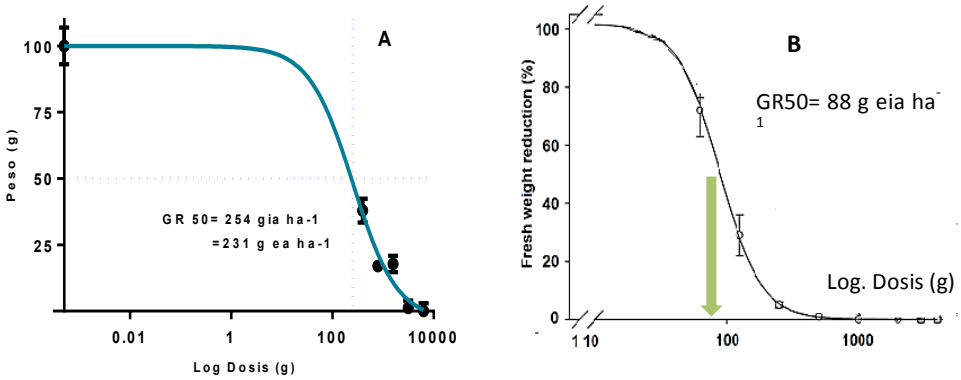


Figura 20. Curva dosis-respuesta y GR_{50} con glifosato sobre *Chloris elata*, biotipo "Sáenz Peña" (A) y biotipo S (B) a los 21 DDA.

Estos resultados coinciden parcialmente por lo expresado por Plácido *et al.* (2013) al mencionar a la especie con tolerancia a glifosato y Bracamonte *et al.*(2017) y Brunharo. *et al*., (2016) como resistente al mismo herbicida.

Evaluación en la eficacia de control de *Chloris elata* con haloxifop-p-metil

Aunque la evaluación visual a los 10 DDA de los tratamientos postemergentes con haloxifop-p-metil mostro un 100 % de mortalidad solo en la dosis más alta, a los 21 DDA fue posible notar una alta efectividad de control entre las dosis 27 y 216 g.i.a. ha^{-1}, alcanzando eficacias entre 83 y 100% de mortalidad. Con la menor dosis no se observó mortalidad (0 % de control; Figura 21 y 22).

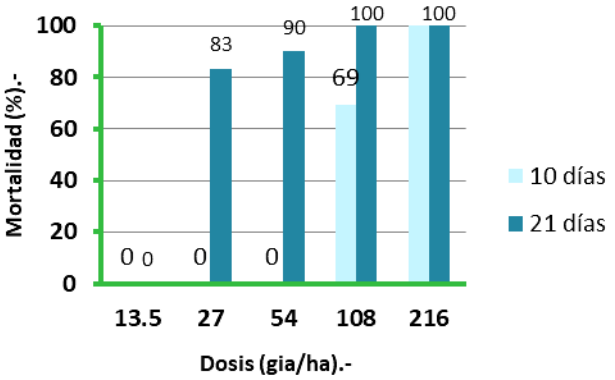


Figura 21. Mortalidad de *Chloris elata* con haloxifop a los 10 y 21 DDA.

Figura 22. Evaluación visual de control de *Chloris elata* con haloxifop a los 21 DDA.

Este efecto retardado en el accionar del herbicida es respaldado por lo observado en la DL_{50} a los 10 DDA (188 g.i.a. ha^{-1}, dato no mostrado) que representa tres veces el valor de la dosis recomendada por la empresa, hasta llegar a los 21 DDA con una DL_{50} de 26.5 g.i.a. ha^{-1} (Figura 23).

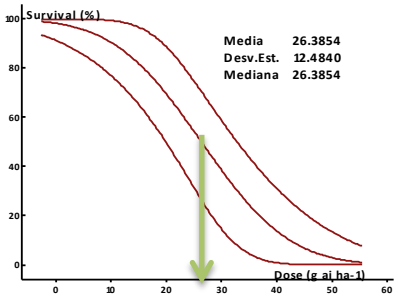


Figura 23. Dosis Letal50 en el control de *Chloris elata* con haloxifop a los 21 DDA.

Considerando el control total de *Chloris virgata* en estadio recomendado, se podría alcanzar con 13.5 g ia ha^{-1} de haloxifop (Figura 14), donde se obtiene un control eficaz con un costo menor en relación a glifosato (6,5 US$ ha^{-1}), Para obtener el mismo resultado con haloxifop en el control de *C. elata* se requiere 108 g.i.a. ha^{-1} (Figura 21), correspondiendo esta dosis a una más alta a la recomendada por la empresa y con un costo de 13 US$/ha, representando el doble de costo en relación a *C.virgata* (Márgenes Agropecuarios, 2016). Esta diferencia observada puede ser debido a que *C.elata* es una especie de tipo perenne, de poseer tolerancia natural alta o ser un biotipo sospechosa de ser resistente a los inhibidores de la ACCASA, lo que sugiere seguir evaluando este biotipo en el futuro.

Evaluación de la eficacia de control de *chloris elata* con herbicidas preemergentes

A los 10 DDA y 21 DDA de los herbicidas preemergentes se pudo observar que Ligate (sulfometuron + clorimuron etil) (L) tuvo un control deficiente alcanzando diclosulam (D) e imazetapir (I) una mejor efectividad (Figura 27 y 28). Los resultados expresaron

que aunque disminuyó levemente el porcentaje de control a los 21 DDA, no hubo diferencias estadísticas significativas en la efectividad de los tratamientos herbicidas en ambos momentos de evaluación (Figura 24).

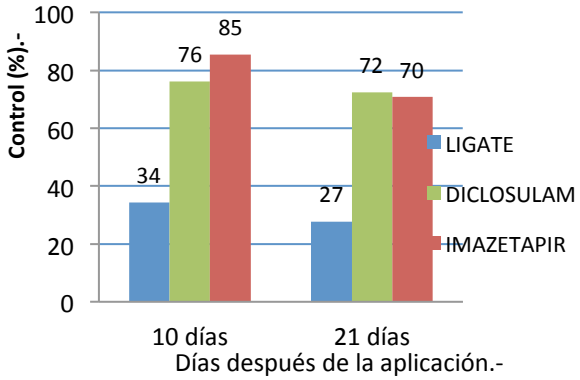


Figura 24. Porcentaje de control con herbicidas preemergentes a 10 y 21 DDA.

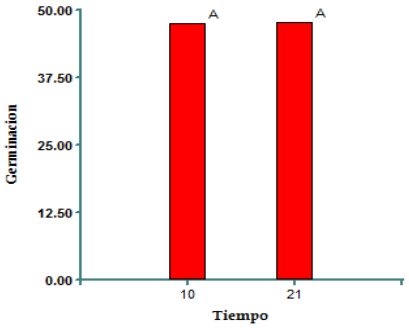


Figura 25. Medias y diferencia significativa entre días después de las aplicaciones con herbicidas preemergentes en el control de *Chloris elata* mediante prueba LSD. Medias con una letra común no son significativamente diferentes (p > 0,05).

La baja eficacia del tratamiento con Ligate en el control de *C. elata* se corroboró al no observarse diferencias estadísticas significativas con el control. El bajo desempeño de Ligate (Figura 21) confirma lo relatado por productores y técnicos de la zona de Sáenz Peña y podría atribuirse al carácter perenne de esta especie, ya que según el marbete comercial indica que el producto está especialmente recomendado para el control de malezas de hoja ancha y gramíneas anuales. Los resultados obtenidos con este herbicida no lo hace apto para utilizarlo en campos invadidos con *C. elata* ni su uso en pre emergencia, pues no alcanzó un nivel de control mínimo que pueda asegurar un cierre del surco limpio por parte de la soja.

Considerando los tratamientos con imazetapir y diclosulam, (Figura 29), la eficacia de estos herbicidas, aunque superiores en relación al traamiento con Ligate, no superaron ninguno el 72% de control. El escape de control observado en germinación (28%)

puede ser atribuido a la alta adsorción de los herbicidas en el sustrato y además del carácter perenne de la especie que podría tornar más difícil su control. Estos resultados abren interrogantes para la realización de más ensayos para validar los datos obtenidos.

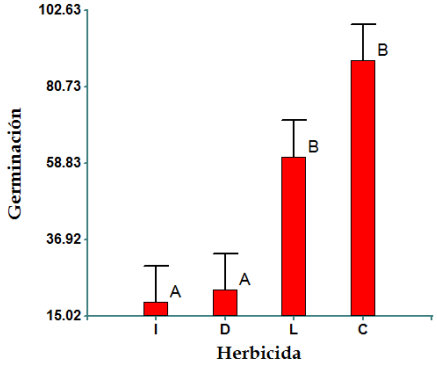


Figura 26. Medidas y diferencias significativas entre herbicidas preemergentes mediante prueba LSD. Medias con una letra común no son significativamente diferentes (p > 0,05).

La opción de uso de los tratamientos con imazetapir y diclosulam, además de no mostrar diferencias estadísticas significativas en cuanto a su accionar y residualidad a los 21 DDA, también muestran un costo por hectárea similar, alcanzando valores de 15,4 y 14,4 US$ ha^{-1} respectivamente (Margenes Agropecarios, 2016).

CONCLUSIONES

Principales

- Biotipos de *Chloris virgata* y *Chloris elata* colectados en Argentina presentan resistencia con un valor de FE 4.09 y 2.6 con respecto al biotipo S, respectivamente. Estos resultados con *Chloris virgata* confirman el primer caso de resistencia de esta especie a este herbicida en Argentina.
- Los herbicidas haloxifop-p-metil en postemergencia y diclosulam e imazetapir en preemergencia constituyen alternativas eficaces en el control de los biotipos *C.elata* y *C.virgata* en Argentina.

Secundarias

- *Chloris virgata*, es sensible al herbicida haloxifop a partir de dosis igual o superior a 13.5 gia/ha en el estado fenológico de 3-4 hojas de la maleza. En estado de macollaje se

obtienen valores apropiados de control con la utilización de dosis comercial recomendada.

•Para lograr un control de 95% con glifosato en macollaje de *Chloris virgata* representa un incremento de casi **33 %** en el costo de control en relación a aplicaciones en premacollaje.

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CAPÍTULO VII

CONCLUSIONES

Las conclusiones obtenidas de:
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Estudios de manejo alterntivo

Son:

- Poblaciones de *P. histerophorus* presentes en Cuba y Republica Dominicana presentan valores altos de resistencia que están determinados por el número de mecanismos de resistencia (TSR y NTSR) que poseen las diferentes poblaciones evaluadas.

- Estudios morfológicos y moleculares permitió identificar a *Choris barbata* Sw, *choris ciliata* Sw y *Chloris elata* Desv. como las especies presentes en huertos de citrus en Cuba.

- Poblaciones de *Choris barbata* y *choris ciliata* presentes en huertos de Cuba presentan tolerancia innata al herbicida glifosato.

- *C. elata* presente en huertos de Cuba presenta resistencia a glifosato, con mecanismos de resistencia fuera del sitio de acción (reducida absorción y translocación) y en el sitio de acción (mutación Pro-106-Ser). Estos resultados confirman el primer caso de resistencia de esta especie a este herbicida en Cuba.

- Poblaciones d*e Choris barbata* presentes en huertos de lima persa en Mexico

presentan evolución de tolerancia a resistencia al herbicida glifosato mediante el cambio de aminoácido de Pro a Ser en la posición 106 del gen EPSPS y translocación alterada como un mecanismo de resistencia en desarrollo fuera del sitio de acción. Estos resultados confirman el primer caso de resistencia de esta especie a este herbicida en Cuba.

- Biotipos de *Chloris virgata* y *Chloris elata* colectados en Argentina presentan un nivel de resistencia de 4.09 y 2.6 con respecto al biotipo S, respectivamente. Estos resultados con *Chloris virgata* confirman el primer caso de resistencia de esta especie a este herbicida en Argentina.

- Los herbicidas haloxifop-p-metil en postemergencia y diclosulam e imazetapir en preemergencia constituyen alternativas eficaces en el control de los biotipos *C.elata* y *C.virgata* en Argentina.

CAPÍTULO VII

PUBLICACIONES

Glyphosate-Resistant *Parthenium hysterophorus* in the Caribbean Islands: Non Target Site Resistance and Target Site Resistance in Relation to Resistance Levels

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Glyphosate has been the most intensely herbicide used worldwide for decades, and continues to be a single tool for controlling weeds in woody crops. However, the adoption of this herbicide in a wide range of culture systems has led to the emergence of resistant weeds. Glyphosate has been widely used primarily on citrus in the Caribbean area, but a study of resistance in the Caribbean islands of Cuba and the Dominican Republic has never been carried out. Unfortunately, *Parthenium hysterophorus* has developed glyphosate-resistance in both islands, independently. The resistance level and mechanisms of different *P. hysterophorus* accessions (three collected in Cuba (Cu-R) and four collected in the Dominican Republic (Do-R) have been studied under greenhouse and laboratory conditions. In *in vivo* assays (glyphosate dose causing 50% reduction in above-ground vegetative biomass and survival), the resistance factor levels showed susceptible accessions (Cu-S ≥ Do-S), low-resistance accessions (Cu-R3 < Do-R4), medium-resistance accessions (Do-R3 < Cu-R2 < Do-R2) and high-resistance accessions (Do-R1 < Cu-R1). In addition, the resistance factor levels were similar to those found in the shikimic acid accumulation at 1000 μM of glyphosate (Cu-R1 ≥ Do-R1 > Do-R2 > Cu-R2 > Do-R3 > Do-R4 > Cu-R3 >> Cu-S ≥ Do-S). Glyphosate was degraded to aminomethylphosphonic acid, glyoxylate and sarcosine by >88% in resistant accessions except in Cu-R3 and Do-R4 resistant accessions (51.12 and 44.21, respectively), whereas a little glyphosate (<9.32%) was degraded in both susceptible accessions at 96 h after treatment. There were significant differences between *P. hysterophorus* accessions in the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) activity enzyme with and without different glyphosate rates. The R accessions showed values of between 0.026 and 0.21μmol μg^{-1} TSP protein min^{-1} basal EPSPS activity values with respect to the S (0.024 and 0.025) accessions. The same trend was found in the EPSPS enzyme activity treated with glyphosate, where a higher enzyme activity inhibition (glyphosate μM) corresponded to greater resistance levels in *P. hysterophorus* accessions. One amino acid substitution was found at position 106 in EPSPS, consisting of a proline to serine change in Cu-R1, Do-R1 Do-R2. The above-mentioned results indicate that high resistance values are determined by the number of defense mechanisms (target-site and non-target-site resistance) possessed by the different *P. hysterophorus* accessions, concurrently.

Keywords: *P.hysterophorus*, target-site and non-target-site mechanisms, resistance levels, glyphosate

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Keywords: *P. hysterophorus*, target-site and non-target-site mechanisms, resistance levels, glyphosate

INTRODUCTION

Herbicide resistance is an evolutionary phenomenon that allows resistant weed biotypes to be exposed to the normal dose of a herbicide undergoing any suffering growth alterations (Fernández et al., 2016). This biological phenomenon is favored by intensive herbicide applications with the same active ingredient or with the same mode of action (Neve et al., 2014; Evans et al., 2016). Glyphosate weed resistance is one of the world's most interesting cases, 35 glyphosate-resistant species have been detected and characterized (mainly using test dose response curves and shikimic acid accumulation) up to date (Heap, 2016).

Glyphosate ((N-phosphonomethyl)-glycine) is a post-emergent herbicide that is non-selective, highly systemic and widely used for weed control around the world (Franz et al., 1997; Székács and Darvas, 2012). It is well metabolized in plants and slow-acting with visible phytotoxic symptoms in sensitive plants at 10–20 days after application (Amrhein et al., 1980; Shingh and Shaner, 1998; Monquero et al., 2004). It inhibits the shikimate pathway by inhibiting 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which catalyzes the synthesis reactions of aromatic amino acids involved in the formation of essential proteins in plants (Sammons and Gaines, 2014).

Glyphosate resistance selection is due to two different mechanisms known as non-target site resistance (NTSR) and target site resistance (TSR) (Shaner et al., 2012; Sammons and Gaines, 2014). NTSR involves a reduced rate of herbicide in the meristem tissues due to limited absorption/translocation, and/or sequestration of the herbicide into compartments such as vacuoles (Michitte et al., 2007; Ge et al., 2012; Vila-Aiub et al., 2012). Metabolic pathways capable of degrading the herbicide to non-toxic compounds in plants also belong to these group mechanisms (De Prado and Franco, 2004; Cruz-Hipólito et al., 2009, 2011; Busi et al., 2011; de Carvalho et al., 2012; González-Torralva et al., 2012; Alcántara-de la Cruz et al., 2016a). TSR has been produced by one or more mutations in the DNA sequence (González-Torralva et al., 2014; Sammons and Gaines, 2014; Fernández et al., 2015; Yu et al., 2015), or by the overexpression of the EPSPS protein by gene amplification (Gaines et al., 2010; Salas et al., 2012, 2015).

When growers reported noticing any deficiency in their weed control, they usually increased the glyphosate doses, which increased the pressure selection as well as triggering the acquisition of a second resistance mechanism (Jasieniuk et al., 1996; González-Torralva et al., 2012). Then, the level of weed resistance to glyphosate increased (Bostamam et al., 2012).

Ragweed parthenium (*Parthenium hysterophorus* L.) is a troublesome annual weed of the *Asteraceae* family that is native to the Gulf of Mexico and other Latin American countries (Rosario et al., 2013). Its prolific seed production (130,000–200,000 seeds m^{-2}), as well as the seeds's ability to persist in the soil and germinate over a wide range of temperatures, have contributed to the widespread distribution of ragweed parthenium in perennial and annual crops (orchards, citrus, soybean, corn) as well as in surrounding areas (Joshi, 1991; Pandey et al., 2003; Navie et al., 2004; Adkins and Shabbir, 2013). In addition, the subtropical environment of the Caribbean Islands (Cuba and Dominican Republic) allows year-round germination, growth, and reproduction of ragweed parthenium, which also contributes to its widespread distribution in the region. Glyphosate has been used repeatedly in perennial crop areas and fallow fields in the Caribbean Islands for many years to manage ragweed parthenium and other troublesome weeds. However, growers have recently observed reduced ragweed parthenium control with single or multiple glyphosate applications. Previous reports have documented glyphosate-resistant ragweed parthenium in Colombia (Rosario et al., 2013), Florida (southeast US) (Fernandez, 2013) and Dominican Republic (Jimenez et al., 2014), but in these three cases the causes of resistance to glyphosate have been inconclusive.

The main objective of this work is a survey of *P. hysterophorus* in Cuba and the Dominican Republic that had never been done before. The specific objectives were to determine (1) the level of glyphosate resistance of different accessions; (2) the possible NTSR and TSR mechanisms involved; and (3) to find out if the resistance genes may also increase the multiplicative or additive resistance levels in *P. hysterophorus*.

MATERIALS AND METHODS

Plant Material

In 2013, mature *P. hysterophorus* seeds were collected from plants not controlled with glyphosate at doses normally used (2 L ha^{-1}; 720 g ae ha^{-1}) in areas with perennial crops in two Caribbean Islands. Seeds from Cu-S and Do-S accessions never exposed to glyphosate were collected from adjacent areas and used as a reference control (**Table 1**). Seeds collected from 25 mature plants were stored under laboratory conditions (25°C) for 2 weeks and then placed in paper bags at 4°C. Approximately 300 seeds of these accessions were sown directly into trays (40 × 60 × 15 cm), containing a mixture of sand and peat (2:1, v/v) and placed in a greenhouse at 28/20°C day/night under a 16 h photoperiod with 850 µmol m^{-2} s^{-1} photon flux density, and 80% relative humidity. At the four leaf stage plants of all accessions were treated with glyphosate at 720 g ae ha^{-1} using a laboratory spray chamber equipped with a flat fan nozzle (TeeJet 8002 EVS) with a total output volume of 200 L ha^{-1} water at

TABLE 1 | History of different *P. hysterophorus* accessions used in this study.

Accessions[a]	Location	Crop	Glyphosate[b] (time of applications per year), number of application years
Cu-R1	Ceiba	Orchards[c]	720 (2 or 3 times), >10
Cu-R2	Ceiba	Citrus[c]	720 (1 time), >10
Cu-R3	Arimao	Citrus	720 (2 times), unknown
Cu-S	Arimao	Road trails	No herbicide treatment
Do-R1	Villa Altagracia	Citrus[c]	900 (2 times), >15
Do-R2	San Cristobal	Citrus	900 (2 times), >15
Do-R3	Monseñor Nouel	Citrus	720 (2 times), >10
Do-R4	Maria T. Sánchez	Orchards	720 (1 time), >10
Do-S	Maria T. Sanchez	Road trails	No herbicide treatment

[a]*Cu, P. hysterophorus harvested in Cuba; Do, P. hysterophorus harvested in Dominican Republic;* [b]*glyphosate g ae ha^{-1};* [c]*the last application was performed manually for every plant.*

a pressure of 200 kPa. Four weeks after glyphosate treatment plant survival of the resistant accessions was estimated, and seed produced from surviving plants was collected and stored in paper bags for all subsequent trials. In the case of susceptible accessions (Cu-S and Do-S), no plant survival was observed 4 weeks after glyphosate treatment.

Dose-Response Assay

Seeds of putative resistant (Cu-R1, Cu-R2, Cu-R3, Do-R1, Do-R2, Do-R3, and Do-R4) and susceptible (Cu-S and Do-S) of the *P. hysterophorus* accessions were germinated in trays (12 × 12 × 6 cm) containing the same substrate as described before and placed in a growth chamber of similar environmental conditions controlled as before. One week after germination, individual seedlings were transplanted into pots (6 × 6 × 8 cm) and grown under fluctuating 30/20°C day/night with a 14 h photoperiod and 850 μmol m^{-2} s^{-1} photon flux density, and 80% relative humidity. As glyphosate (EPSPS inhibitor) is used in early post-emergence, at the four leaf stage, resistant and susceptible *P. hysterophorus* seedlings were treated with increasing glyphosate doses: 0, 31.25, 62.5, 125, 250, 500, 1000, 2000, 4000, and 8000 g ae ha^{-1} (Roundup Energy 45% w/v, SL, Monsanto Spain). The experiment were conducted with 10 replications (one plant pot^{-1}) of each accession per herbicide dose, and the experiments were repeated twice. Thirty days after herbicide treatment, herbicide effects on plant survival (LD) and above-ground vegetative biomass (GR) were assessed.

Leaf Segment Shikimate Accumulation Assay

Leaf segments (50 mm diameter) were harvested from the youngest fully expanded leaf from a batch of 15 plants per *P. hysterophorus* accessions at the 4–6 leaf stage (Hanson et al., 2009). Approximately 50 mg of fresh tissue was transferred to 2 mL Eppendorf tubes containing 1 mL of 1 mM $NH_4H_2PO_4$ (pH 4.4). Glyphosate was added to the tubes at the following concentrations: 0, 0.1, 0.5, 1, 5, 10, 50, 100, 200, 400, 500, 600, and 1000 μM. The Eppendorf tubes were incubated in a growth chamber during 24 h under the previously described conditions. After 24 h, the tubes were stored at −20°C until analysis. Eppendorf tubes were removed from the freezer and thawed at 60°C for 30 min. Two hundred and fifty micro liters of 1.25 N HCL was added to each tube, and placed at 60°C for 15 min. A 125 μL aliquot from each tube was pipetted into a new 2 mL Eppendorf tube, and 500 μL of periodic acid and sodium metaperiodate (0.25% [wt/v] each) was added. They were incubated at room temperature for 90 min, after which 500 μL of 0.6 N sodium hydroxide and 0.22 M sodium sulfite was added. The contents of all tubes were transferred to glass vials. Samples were measured in a spectrophotometer at 380 nm within 30 min. For each glyphosate concentration and accession, three replications were stablished and repeated twice.

^{14}C Glyphosate Absorption and Translocation

Absorption and translocation study was carried out following the methodology proposed by Cruz-Hipólito et al. (2011) The ^{14}C-glyphosate was mixed with commercially formulated glyphosate to prepare a solution with a specific activity of 0.834 kBq μL^{-1} and a glyphosate concentration of 1.8 g ae L^{-1} (360 g ae ha^{-1} in 200 L). *P. hysterophorus* plants at 4-leaf stage were treated with the radiolabeled herbicide by applying one droplet of 1 μL of glyphosate solution (0.834 kBq μL^{-1}) on the adaxial surface of the second leaf in each plant using a micropipette (LabMate). The ^{14}C-glyphosate unabsorbed in the treated leaf was removed with 3 mL of water: acetone solution (1:1, v/v) 96 h after droplet application. Preliminary assays with two accessions (Cu-R1 and Cu-S) studied had revealed that the glyphosate absorption leveled-off at 96 h after the droplet applications. The rinsate was mixed with 2 mL of scintillation liquid and analyzed by liquid scintillation spectrometry (LSS) (Scintillation Counter, Beckman LS 6500, Fullerton CA). The plants were separated into the treated leaf, rest of the shoot and root after being placed in cellulose cones. The plant tissue was dried at 60°C over 96 h and combusted in a biological sample oxidizer (Packard Tri Carb 307, Perkin-Elmer, Waltham, MA). The $^{14}CO_2$ evolved was trapped and counted in 18 mL of a mixture of Carbo-Sarb E and Permafluor (9:9, v/v) (Perkin-Elmer). Thus, over 95% of the total radioactivity applied was recovered. There were five replications and the experiment was arranged in a completely randomized design, and repeated twice. The proportion of absorbed herbicide was expressed as:

$$[\%\ \text{absorbed} = (\text{kBq in combusted tissue}/(\text{kBq in combusted tissue} + \text{kBq in leaf washes})) \times 100].$$

Glyphosate Metabolism

P. hysterophorus plants were treated with a glyphosate rate of 360 g ae ha^{-1} at 4–6 leaf stage. At 96 h after treatment (HAT), glyphosate and its metabolites, i.e., AMPA (aminomethylphosphonic acid), glyoxylate and sarcosine, were determined by reversed-polarity capillary electrophoresis following the methodology described by Rojano-Delgado

et al. (2010). The calibration equations were established using non-treated plants and known concentrations of glyphosate and its metabolites, which were determined from their enclosed areas under the peaks in the electropherogram. The average value for the amount of glyoxylate naturally produced by the plant was subtracted from the average of the produced or reduced amount after treatment of each accession (Rojano-Delgado et al., 2010). The experiment was arranged in a completely randomized design with four replications per accession and repeated three times.

EPSPS Enzyme Activity Assays

The enzyme extraction was conducted according to the protocol described by Dayan et al. (2015). Five gram of the leaf tissue of all *P. hysterophorus* accessions **(Table 1)** were ground to fine powder in a chilled mortar. Immediately after that, the powdered tissue was transferred to tubes containing 100 mL of cold extraction buffer (100 mM MOPS, 5 mM EDTA, 10% glycerol, 50 mMKCl and 0.5 mM benzamidine) containing 70 μL of β-mercaptoethanol and 1% in polyvinylpolypyrrolidone (PVPP). Samples were stirred and subsequently centrifuged for 40 min (18,000 g) at 4°C. The supernatant was decanted into a beaker using a cheesecloth. $(NH_4)_2SO_4$ was added to the solution to obtain 45% (w/v) concentration, with stirring during 30 min. After that, the mix was centrifuged at 20,000 g for 30 min at 4°C. The previous step was repeated to precipitate the protein in the extracts but in that case with a $(NH_4)_2SO_4$ concentration of 80% (w/v) stirring for 30 min. Finally, they were centrifuged at 20,000 × g for 30 min at 4°C.

All the pellets were dissolved in 3 mL of extraction buffer and dialyzed in 2 L of dialysis buffer (30 mm, 1000-MWC dialysis tubing at 4°C on a stir plate) over 12 h. The protein concentrations were determined by Bradford assay (Bradford, 1976).

The assay for the determination of EPSPS activity followed the methodology described by Dayan et al. (2015) using the EnzCheck phosphate assay Kit (Invitrogen, Carlsbad, CA) to determine the inorganic phosphate release. The EPSPS activity from the nine accessions was determined in the presence and absence of glyphosate. The glyphosate concentrations used were: 0, 0.1, 1, 10, 100, and 1000 μM to determine the enzyme activity inhibition (I_{50}). The assay buffer was composed of 1 mM $MgCl_2$, 10% glycerol, and 100 mM MOPS, 2 mM sodiummolybdate and 200 mM NaF. The experiments were conducted with three replications of each accession per glyphosate concentration and repeated three times. EPSPS enzyme activity was expressed as percentage of enzyme activity in presence of glyphosate respect to the control (without glyphosate).

EPSP Synthase Gene Sequencing

For RNA extraction 100–200 mg of young leaves were taken from plants of each *P. hysterophorus* accession, and stored at −80°C for the extraction of RNA. Their tissue was ground in liquid nitrogen in a STAR-BEATER 412–0167 mill (VWR International Eurolab S.L., Barcelona, Spain). Total RNA was isolated from leaves as described by Pistón (2013), and the amount and quality were determined in a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Walthman, MA, USA). The synthesis to cDNA was from total RNA being adjusted to the same concentration in all the samples (50 ng μL^{-1}). An iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Inc. CA, USA) at 40 μL reaction volume was used following the manufacturer's instructions.

The PCR reactions were carried out with cDNA samples from each of the accession using the primers *Bidens*-F10 (5′- GGTTGTGGYGGTVTRTTTCC-3′) and *Bidens*-R11 (5′-GTCCCAASTATCACTRTGTTC-3′) based on EPSPS gene sequences described previously (Alcántara-de la Cruz et al., 2016b). PCR conditions were also as described (Alcántara-de la Cruz et al., 2016b). The PCR on cDNA amplified fragments of 462 bp in length, comprising the region of Thr-102 and Pro-106, which corresponds to the sequence of the EPSPS gene of *Arabidopsis* Klee et al. (1987), in which point mutations conferring resistance to glyphosate have been associated (Sammons and Gaines, 2014; Yu et al., 2015).

The PCR fragments were cloned in the pGEM®-T Easy Vector System (Promega Biotech Ibérica, SL, Madrid, Spain) and transformed into competent cells of *E. coli* DH5α (Promega). Transformation was confirmed through PCR using the M13F and M13R primers as described (Alcántara-de la Cruz et al., 2016b). The colonies containing the length of the fragment were sequenced by the STABVIDA sequencing service (Caparica, Portugal). Five biological samples were used per accession providing 15 clones in all for each one. The quality and assembly of cDNA sequences and consensuses were determined employing the programs of SeqMan Pro™ versión 11(DNASTAR; Wisconsin, USA) and Geneious® versión 8.1.8 (Biomatters Ltd, Auckland, New Zealand). The multiple sequences were aligned by means of the Muscle algorithm incorporated into SeqMan Pro versión 11.

Data Analysis

Dose-Response and EPSPS enzyme activity data were subjected to non-linear regression analysis (Seefeldt et al., 1995; Burgos et al., 2013) using a three-parameter log-logistic equation (Equation 1) to determine the glyphosate dose causing 50% reduction in above-ground vegetative biomass (GR_{50}), 50% mortality (LD_{50}), and inhibition of EPSPS activity by 50% (I_{50}).

$$Y = \{[(d) / (1 + (x/g)^b)]\} \quad (1)$$

Where Y is the EPSPS activity, survival or above-ground biomass at herbicide x dose, d is the coefficient corresponding to the upper asymptote, b is the slope of the curve, and g is the herbicide rate at the point of inflection halfway (i.e., LD_{50}, GR_{50}, I_{50}).

Regression analyses were conducted using the *drc* package (Ritz et al., 2015) for the statistical environment R (R 3.2.4; R Core Team, 2015). Resistance indices were computed as R-to-S GR_{50} LD_{50}, or I_{50} ratios. To test for a common GR_{50}, LD_{50}, or I_{50} for R and S accessions, i.e., Resistance Index equals to 1, a lack-of-fit test was used to compare the model consisting of curves with accessions-specific g values with a reduced model with common g (Ritz et al., 2015).

Analysis of variance (ANOVA) was conducted using Statistix 9.0 (Analytical Software, USA) to test for differences between

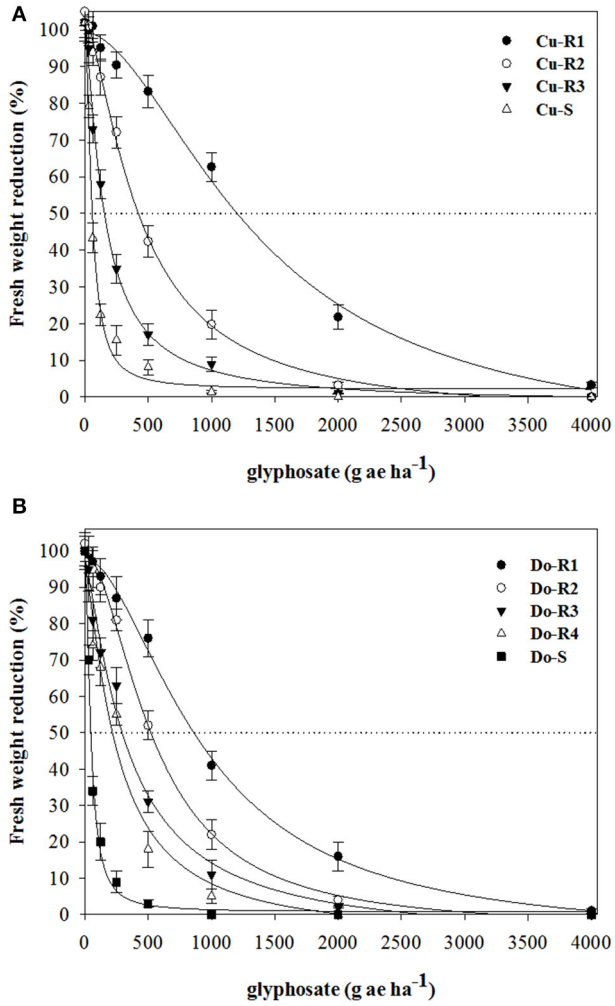

FIGURE 1 | Shoot biomass in glyphosate-resistant and susceptible *P. hystherophorus* accessions from Cuba (A) and Dominican Republic **(B)** 30 days after treatment. Symbols denoted mean ($n = 10$) ± standard errors of the mean.

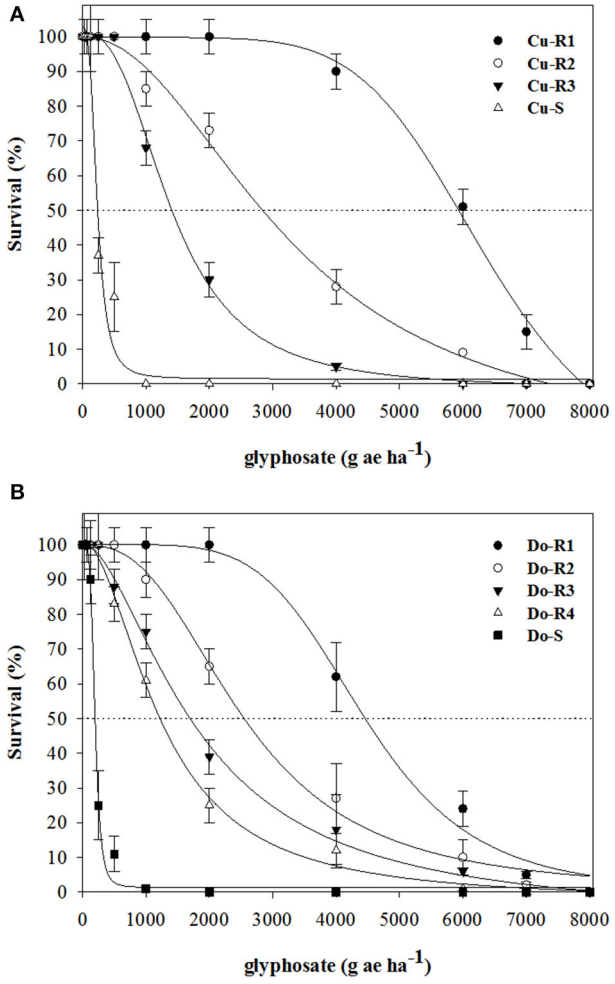

FIGURE 2 | Survival plants in glyphosate-resistant and susceptible *P. hystherophorus* accessions from Cuba (A) and Dominican Republic **(B)** 30 days after treatment. Symbols denoted mean ($n = 10$) ± standard errors of the mean.

R and S accessions in shikimate accumulation at 1000 μM glyphosate in the leaf segment; and proportion of the different glyphosate metabolites; proportion of applied ^{14}C-glyphosate taken up by leaves, and proportions of absorbed ^{14}C-glyphosate remaining in the treated leaf, translocated to roots and to the rest of the plant at 96 HAT; and basal enzyme activity. Percentage data were previously transformed (arcsine of the square root) to meet model assumptions. Model assumptions of normal distribution of errors and homogeneous variance were graphically inspected. When needed, differences between means were separated using the Tukey HSD test.

RESULTS

Physiological Studies

Dose-response assays showed the existence of the first case of glyphosate-resistant weeds in the Caribbean (Cuba and Dominican Republic). The two susceptible weeds (Cu-S and Do-S) had similar susceptibility levels (**Figures 1**, **2**; **Table 2**). The *P. hyterophorus* accessions from Cuba island had resistance index (RI) values (based on the GR_{50} and LD_{50} values) that ranged from 2.7 to 24.6, and 6.1 to 27.5 fold resistance, respectively, while on Dominican Republic island values were between 5.4 to 20, and 6.3 to 22.7 fold resistance, respectively (**Table 2**).

The fact that plants treated with glyphosate increase shikimic acid accumulation in leaf disks due to the inhibition of EPSPS activity led us to carry out the experiment depicted in **Figures 3A,B**. Considering the values obtained *in vivo* (GR_{50} and LD_{50}) and the shikimic acid accumulation in leaf disks at 1000 μM of glyphosate, the resistance order of the *P. hystherophorus* accessions was Cu-R1 ≥ Do-R1 > Do-R2 > Cu-R2 > Do-R3 > Do-R4 > Cu-R3 >> Cu-S ≥ Do-S. There were significant differences at 1000 μM glyphosate between R and S accessions of Cuba ($p = 0.0013$, $DF = 3$, $n = 12$) and Dominican Republic ($p = 0.0008$, $DF = 4$, $n = 15$).

TABLE 2 | Parameters of the log-logistic equations used to calculate the glyphosate rates required for 50% survival (LD_{50}) and reduction fresh weight (GR_{50}) of the different accessions of *P. hyterophorus* from Cuba and Dominican Republic.

Accessions	Survival[a] (%)						Fresh weight reduction[b] (%)					
	d	*b*	R^2	LD_{50} (g ae ha^{-1})	RI	*p*	*d*	*b*	R^2	GR_{50} (g ae ha^{-1})	RI	*p*
Cu-R1	99.8	6.1	0.98	6364 ± 122	27.5	<0.0001	99.4	1.8	0.99	1370 ± 191	24.5	<0.0001
Cu-R2	98.9	2.9	0.99	2794 ± 90	12.0	<0.0001	103.0	1.5	0.95	437 ± 28	7.8	<0.0001
Cu-R3	100.9	2.6	0.99	1415 ± 55	6.1	<0.0001	103.3	1.3	0.96	151 ± 13	2.7	0.003
Cu-S	102.7	3.1	0.97	232 ± 23	–	–	103.2	1.7	0.98	56 ± 6	–	–
Do-R1	100.1	5.1	0.96	4456 ± 76	22.7	<0.0001	98.2	1.8	0.98	939 ± 25	20.0	<0.0001
Do-R2	99.9	2.7	0.98	2550 ± 92	13.0	<0.0001	99.6	1.8	0.99	547 ± 30	11.6	<0.0001
Do-R3	100.7	1.7	0.99	1821 ± 63	9.3	<0.0001	97.9	1.3	0.99	339 ± 27	7.2	<0.0001
Do-R4	100.9	1.9	0.99	1242 ± 65	6.3	<0.0001	96.4	1.3	0.96	255 ± 33	5.4	<0.0001
Do-S	100.5	4.5	0.97	196 ± 8	–	–	100.6	1.7	0.98	47 ± 4	–	–

[a] *For $Y = \{(d) / [1 + (x/ LD_{50}) \exp b]\}$ Where Y is the survival expressed as a percentage of the untreated control, d is the coefficient corresponding to the upper asymptote, b is the slope of the curve in LD_{50}, LD_{50} is the herbicide rate at the point of inflection halfway, and x is the herbicide dose.*

[b] *For $Y = (d) / [1 + (x/ GR_{50}) \exp b]$ Where Y is the above-ground weight expressed as a percentage of the untreated control, d is the coefficient corresponding to the upper asymptote, b is the slope of the curve in GR_{50}, GR_{50} is the herbicide rate at the point of inflection halfway, and x is the herbicide dose.*

TABLE 3 | ^{14}C-glyphosate absorption (% of recovered radioactivity) and translocation (% of absorbed radioactivity) in the different *P. hysterophorus* accessions at 96 h after treatment (HAT).

Accessions	Absorption[a] ($p = 0.0001, DF = 8, n = 45$)	Translocation		
		Treated leaf ($p = 0.0003, DF = 8, n = 45$)	Rest of shoot ($p = 0.0001, DF = 8, n = 45$)	Root ($p = 0.0004, DF = 8, n = 45$)
Cu-R1	59.3 ± 4.9 BC	77.9 ± 5.7 AB	12.1 ± 2.1 BCD	10.0 ± 2.3 BC
Cu-R2	60.2 ± 2.1 BC	82.4 ± 4.2 A	9.3 ± 1.9 D	8.3 ± 3.4 BCD
Cu-R3	56.8 ± 3.9 C	80.1 ± 3.9 AB	15.7 ± 3.4 B	4.2 ± 1.2 D
Cu-S	82.2 ± 6.7 A	35.5 ± 2.3 C	41.6 ± 6.2 A	22.9 ± 4.8 A
Do-R1	63.1 ± 6.8 B	78.3 ± 6.7 AB	10.5 ± 2.7 CD	11.2 ± 2.1 B
Do-R2	55.9 ± 7.8 C	79.3 ± 3.4 AB	16.2 ± 4.9 B	4.5 ± 1.4 D
Do-R3	60.4 ± 3.7 BC	75.6 ± 5.1 B	14.1 ± 3.8 BC	10.3 ± 3.8 B
Do-R4	58.4 ± 2.3 BC	81.4 ± 6.3 A	12.7 ± 4.3 BCD	5.9 ± 2.7 CD
Do-S	78.8 ± 5.6 A	39.1 ± 1.9 C	37.8 ± 2.3 A	23.1 ± 5.6 A

[a] *Over 95% of the total radioactivity applied was recovered.*

Mean value ($n = 5$) ± standard error. Means on a same column followed by the same letter were not significantly different at $\alpha = 0.05$.

There were marked differences in glyphosate absorption between the resistant and susceptible glyphosate *P. hysterophorus* accessions at 96 h after treatment (HAT) ($p = 0.0001$, $DF = 8$, $n = 45$) (**Table 3**). All accessions obtain maximum absorption at 96 HAT, and the two susceptible accessions absorbed an average of 80.5%, while the resistance accessions absorbed an average of 59.2% of ^{14}C-glyphosate which was recovered.

Translocation assays suggest marked differences at 96 HAT between the Cu-S and Do-S accessions compared to the Cu-R1, Cu-R2, Cu-R3, Do-R1, Do-R2, Do-R3, and Do-R4 ones in treated leaf ($p = 0.0003$, $DF = 8$, $n = 45$), rest of the shoots ($p = 0.0001$, $DF = 8$, $n = 45$), and root ($p = 0.0004$, $DF = 8$, $n = 45$) (**Table 3**). There were no significant differences in translocation between the two susceptible accessions (Cu-S and Do-S) from Caribbean Islands. But there were small significant differences in the resistant accessions (Cu-R1, Cu-R2, Cu-R3, Do-R1, Do-R2, Do-R3, and Do-R4). Nonetheless, the high amount of ^{14}C-glyphosate in each resistant accession remained in the treated leaf. Due to differences in levels of glyphosate resistance between the *P. hysterophous* resistant accessions, we suspect that other mechanisms could be involved (**Tables 2**, **3**, **Figure 3**).

Biochemical Studies

Previous tests demonstrated that the highest glyphosate translocation and metabolism was reached at 96 HAT in the *P. hysterophorus* accessions (unpublished data). There were significant differences at 96 HAT in glyphosate metabolism levels between accessions ($p = 0.0014$, $DF = 8$, $n = 36$). Glyphosate levels decreased, whereas glyphosate metabolites (AMPA, glyoxylate and sarcosine) increased at 96 HAT in the Cu-R1, Do-R1, Do-R2, Cu-R2, and Do-R3 accessions. Higher glyphosate levels remained in the Cu-R3 and Do-R4 (low resistance), and

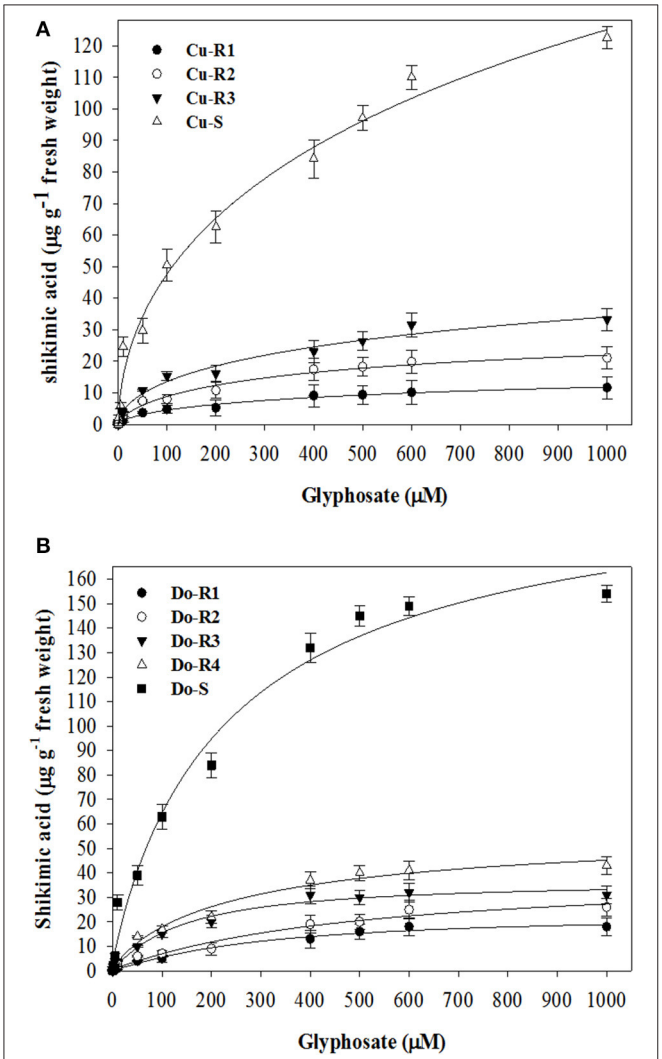

FIGURE 3 | Shikimic acid accumulation in leaf segments of plants from Cuba (A) and Dominican Republic **(B)** accessions of *P. hysterophorus*. Symbols denoted mean ($n = 3$) ± standard errors of the mean.

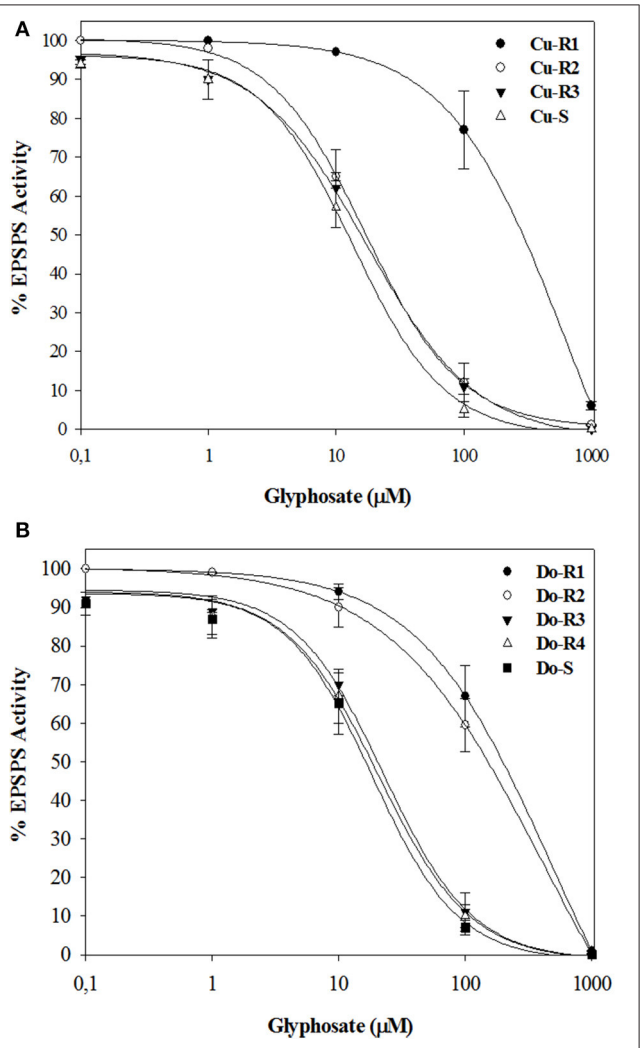

FIGURE 4 | EPSPS enzyme activity expressed as percentage of the untreated control in leaf extracts of plants from Cuba (A) and Dominican Republic **(B)** accessions of *P. hysterophorus*. Symbols denoted mean ($n = 3$) ± standard errors of the mean.

very high one in the Cu-S and Do-S (susceptible) accessions. In these last accessions, sarcosine was not detected (**Table 4**). These results can also explain the low level of resistance of the accession (Cu-R3 and Do-R4) with a single resistance mechanism, while the other glyphosate resistant accessions have at least two mechanisms (**Tables 3**, **4**).

The EPSPS enzymes of all the accession plants were inhibited by glyphosate. The I_{50} (herbicide dose which reduces the enzyme activity to 50%) values were different in all accessions, ranging between approximately 47.65 in Cu-R1, 25.2 in Do-R1, 22.1 in Do-R2, 1.4 in Cu-R2, 1.2 in Do-R3, 1.2 in the Cu-R3, and 1.1-fold resistance in Do-R4 accessions relative to their susceptible accession, respectively (**Figure 4**, **Table 5**). These results were in accordance with the *in vivo* resistance level shown for the different accessions, and suggest that multiple mechanisms in the target-site could be expressed in these accessions.

The basal activity of EPSPS enzyme (without glyphosate) in the resistant accessions was between 0.026 and 0.21 µmol μg^{-1} protein min^{-1}, while the susceptible accessions (Cu-S and Do-S) were lower with 0.024 and 0.025 µmol μg^{-1} protein min^{-1}, respectively (**Figure 5**). There were market differences between accessions in both Cuba ($p = 0.0001$, $DF = 3$, $n = 12$), and Dominican Republic ($p = 0.0002$, $DF = 4$, $n = 15$). The Cu-R1, Do-R1, and Do-R2 exhibited 8.8, 7.2, and 4.8-times higher basal enzyme activities than their susceptible accessions, respectively. For Cu-R2, Do-R3, Do-R4 ,and Cu-R3 accessions the values were similar to those found for their susceptible accessions, respectively.

Molecular Studies

A total of 462 bp of the EPSPS gene of *P. hysterophorus* plants of resistant and susceptible accessions were sequenced. The fragments were aligned and numbered based on a published

TABLE 4 | Glyphosate metabolism expressed as a percentage of total glyphosate and its metabolites in *P. hystherophorus* susceptible and resistant-glyphosate accessions at 96 HAT.

Accessions	Glyphosate ($p = 0.0014, DF = 8, n = 36$)	Metabolites		
		AMPA ($p = 0.0003, DF = 8, n = 36$)	Glyoxylate ($p = 0.0001, DF = 8, n = 36$)	Sarcosine ($p = 0.0002, DF = 8, n = 36$)
Cu-R1	9.80 ± 1.70 D	60.54 ± 1.32 B	18.14 ± 0.32 C	11.52 ± 0.96 A
Cu-R2	21.12 ± 0.93 C	55.31 ± 1.57 B	20.80 ± 0.51 AB	2.77 ± 0.31 E
Cu-R3	73.42 ± 3.63 B	26.14 ± 0.26 C	0.44 ± 0.02 E	ND
Cu-S	91.82 ± 4.81 A	7.68 ± 0.33 E	0.50 ± 0.02 E	ND
Do-R1	11.83 ± 0.74 D	58.94 ± 2.79 B	21.74 ± 0.97 A	7.49 ± 0.27 C
Do-R2	11.37 ± 0.80 D	64.70 ± 2.93 A	18.54 ± 0.83 C	5.39 ± 0.15 D
Do-R3	9.56 ± 0.72 D	60.95 ± 2.71 B	20.36 ± 0.94 B	9.13 ± 0.53 B
Do-R4	71.21 ± 1.06 B	20.05 ± 2.20 D	7.28 ± 0.93 D	1.01 ± 0.71 F
Do-S	90.68 ± 4.39 A	8.86 ± 1.06 E	0.46 ± 0.03 E	ND

Mean value (n = 4) ± standard error. Means on a same column followed by the same letter were not significantly different at α = 0.05.
ND, non-detected; AMPA, aminomethylphosphonic acid.

EPSPS sequence of *Arabidopsis thaliana* (L.) Heynh. (GenBank: CAA29828.1). The resistant accessions of *P. hysterophorus* Cu-R1 from Cuba, and Do-R1 and Do-R2 from Dominican Republic, showed an amino acid substitution at position 106 consisting of a Proline to Serine (**Figure 6**).

TABLE 5 | Parameter estimates of the equation used to calculate the sensitivity of EPSPS enzyme activity to glyphosate in extracts from leaf tissue of the different accessions of *P. hyterophorus* from Cuba and Dominican Republic.

Accessions	*d*	*b*	R^2	I_{50} (μM)[a]	RI	*P*
Cu-R1	100.1	0.9	0.97	646.2 ± 35.8	47.6	<0.0001
Cu-R2	99.8	0.8	0.96	18.9 ± 1.4	1.4	0.1902
Cu-R3	97.0	1.0	0.99	17.4 ± 2.8	1.2	0.2186
Cu-S	96.2	1.2	0.98	13.6 ± 2.2	–	–
Do-R1	100.0	0.8	0.99	468.1 ± 22.0	25.2	<0.0001
Do-R2	100.4	0.7	0.99	410.7 ± 26.1	22.1	<0.0001
Do-R3	94.5	1.2	0.98	22.6 ± 1.5	1.2	0.3714
Do-R4	94.0	1.2	0.96	20.8 ± 6.1	1.1	0.6042
Do-S	93.6	1.2	0.99	18.5 ± 5.7	–	–

[a] *For $Y = \{(d) / [1 + (x/ I_{50}) \exp b]\}$ Where Y is the EPSPS activity, d is the coefficient corresponding to the upper asymptote, b is the slope of the curve in I_{50}, I_{50} is the herbicide rate at the point of inflection halfway, and x is the herbicide dose.*

DISCUSSION

P. hysterophorus is universally recognized for its widespread distribution and high seed production, commonly known as the parthenium weed. Parker (1989) identified two biotypes with different flowering patterns in Mexico (Caribbean area), and they were genetically distinct biotypes (Clermont and Toogoolawah). Moreover, Hanif et al. (2011) found that these two biotypes differed in their morphology and reproductive behavior; in particular, the Toogoolawah biotype shows a greater tendency toward self-pollination, but these biotypes can also present out-crossing. It makes sense that it would reproduce prolifically and that higher resistance levels due to accumulation of multiple mechanisms, by multiple crossings, would proliferate within populations (**Table 6**).

Glyphosate has been used repeatedly in perennial crop areas and fallow fields in the Caribbean Islands for many years to manage *P. hysterophorus* and other troublesome weeds. However, using glyphosate alone without any additional alternative and/or IWM (Integrated Weed Management) led to the emergence of glyphosate-resistant weeds early in the second decade of the 21st century (**Tables 1**, **2**). Herbicide response between different locations depends on local ecological factors, such as a variation in soil type, tillage practices, types of crops, fertilizers, etc., (Shaner and Beckie, 2014; Jussaume and Ervin, 2016). Our results showed different glyphosate resistance levels between the *P. hysterophorus* accessions. This differences could be addressed to the use of different glyphosate formulations and dose rate, the application technique (manual or mechanical) employed by farmers, and the agro environment conditions (Neve et al., 2014; Renton et al., 2014; Jussaume and Ervin, 2016; Matzrafi et al., 2016; Owen, 2016). It has been shown that an increase in the relative humidity and temperature increases the glyphosate absorption, translocation, and toxicity in many weed species (Ge et al., 2011; Hatterman-Valenti et al., 2011; Vila-Aiub et al., 2012; Santos et al., 2016). This research also revealed that the low GR_{50} and LD_{50} values for the susceptible accessions showed that glyphosate has been a very effective tool for farmer for over 15 years, as has been shown in *P. hysterophorus* from Colombia, Dominican Republic, and Florida (Fernandez, 2013; Rosario et al., 2013; Jimenez et al., 2014).

Plants with low levels of GR_{50} and LD_{50} are related to an increased inhibition of EPSPS activity and a greater accumulation of shikimic acid (Shaner et al., 2005; Gaines et al., 2010; Fernández et al., 2015). High levels of resistance (RI) and low shikimic acid accumulation observed in the different *P. hystherophorus* accessions were consistent with those of plants

which have acquired resistance to the addition of more than one NTSR and/or TSR mechanisms, as has been shown in dicotyledonous weed species such as *Amaranthus tuberculatus* (Nandula et al., 2013), *Conyza sumatrensis* (González-Torralva et al., 2014), and several grass weed species (Michitte et al., 2007; de Carvalho et al., 2012; Fernández et al., 2015).

According to Shepherd and Griffiths (2006), a cuticular wax layer provides a protective barrier for a wide range of abiotic stresses (pesticide). Resistant and tolerant plants have displayed a cuticle containing a massive amount of epicuticular wax which forms a nonuniform 3D cover as has been revealed by scanning electron micrographs (De Prado et al., 2005; Wang and Liu, 2007; Rojano-Delgado et al., 2012; Alcántara-de la Cruz et al., 2016a). The limited glyphosate absorption by the resistant *P. hysterophorus* accessions was likely to have been due to differences in outer leaf surfaces. Different translocation can be explained by ^{14}C-glyphosate and/or its metabolite accumulation in the tips of the resistant treated leaves, while ^{14}C was removed from the susceptible treated leaves (**Table 3**). Since the first case of glyphosate resistance was detected in a population of *Lolium rigidum* in Australia (Powles et al., 1998), both previously mentioned mechanisms were considered responsible for this resistance (Wakelin et al., 2004; Michitte et al., 2007; Preston and Wakelin, 2008; de Carvalho et al., 2012; González-Torralva et al., 2012, 2014; Nandula et al., 2013; Fernández et al., 2015). Subsequent studies in the main dicot and monocotyledonous glyphosate-resistant weeds seem to have demonstrated that the main NTSR mechanism involved in their resistance is due to a lesser glyphosate absorption and/or -translocation (Feng et al., 2004; Michitte et al., 2007; de Carvalho et al., 2012; González-Torralva et al., 2012, 2014; Vila-Aiub et al., 2012; Nandula et al., 2013; Adu-Yeboah et al., 2014).

In some plants, the glyphosate degradation to glyoxylate and AMPA is carried out by a glyphosate oxidoreductase (GOX), and the glyphosate degradation to sarcosine and inorganic phosphate by a C–P lyase. These steps have been reported by some authors such as Liu et al. (1991); Komoba et al. (1992); Saroha et al. (1998); Al-Rajab and Schiavon (2010), and Duke (2012) among others. However, only a few works unify these two degradation pathways to explain the glyphosate metabolism in leguminous plants and weeds (de Carvalho et al., 2012; Rojano-Delgado et al., 2012). Some authors consider that metabolism has a low contribution to the resistance or, even more, that it is nonexistent (Saroha et al., 1998; Feng et al., 2004; Duke, 2012; Sammons and Gaines, 2014). However, the fact is that this mechanism involves a decrease in the concentration of the herbicide glyphosate around the target-site, diminishing the EPSPS inhibition rate (Duke, 2012; Sammons and Gaines, 2014; Alcántara-de la Cruz et al., 2016a). The GOX gene that encodes the glyphosate metabolizing enzyme glyphosate oxidoreductase was cloned from *Achromobacter* sp. *strain* LBAA (Barry et al., 1994). Neither plant GOX nor the gene(s) encoding it have been isolated or elucidated. A plant gene encoding GOX might be useful in genetically engineering crops and weed resistance development (Duke, 2012; Rojano-Delgado et al., 2012). Some researchers have proposed additive effects of concurrent glyphosate resistance mechanisms in the same weed species (Gaines et al., 2010; Yu et al., 2010; Bostamam et al., 2012; Rojano-Delgado et al., 2012), which would explain the difference in the resistance between accessions keeping the same percentage of metabolic degradation (**Table 6**). However, genetic basic controlling absorption/translocation and/or metabolism including genes involved have not been identified so far (Yuan et al., 2006; Delye, 2013; Délye et al., 2013). This could be a highly promising research area in the future.

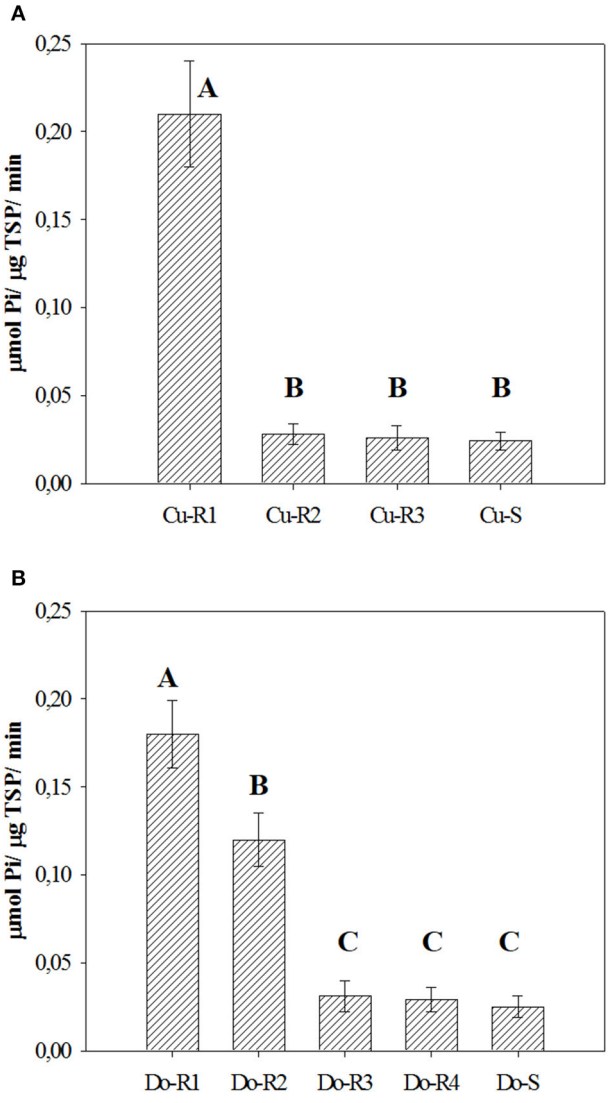

FIGURE 5 | Basal EPSPS activity for glyphosate-susceptible and resistant from Cuba (A) and Dominican Republic **(B)** accessions of *P. hysterophorus*. Vertical bars are ± standard errors of the mean. Means by the same letter were not significantly different at $\alpha = 0.05$.

Taking into account these results, resistance could be associated with target enzyme overexpression. Some species as ryegrass (Yu et al., 2007; Dayan et al., 2012) have shown differences in the basal EPSPS enzyme activity as a consequence of the EPSPS gene overexpression. However, in the *L. perenne* spp. *multiflorum* population from Arkansas, no differences were

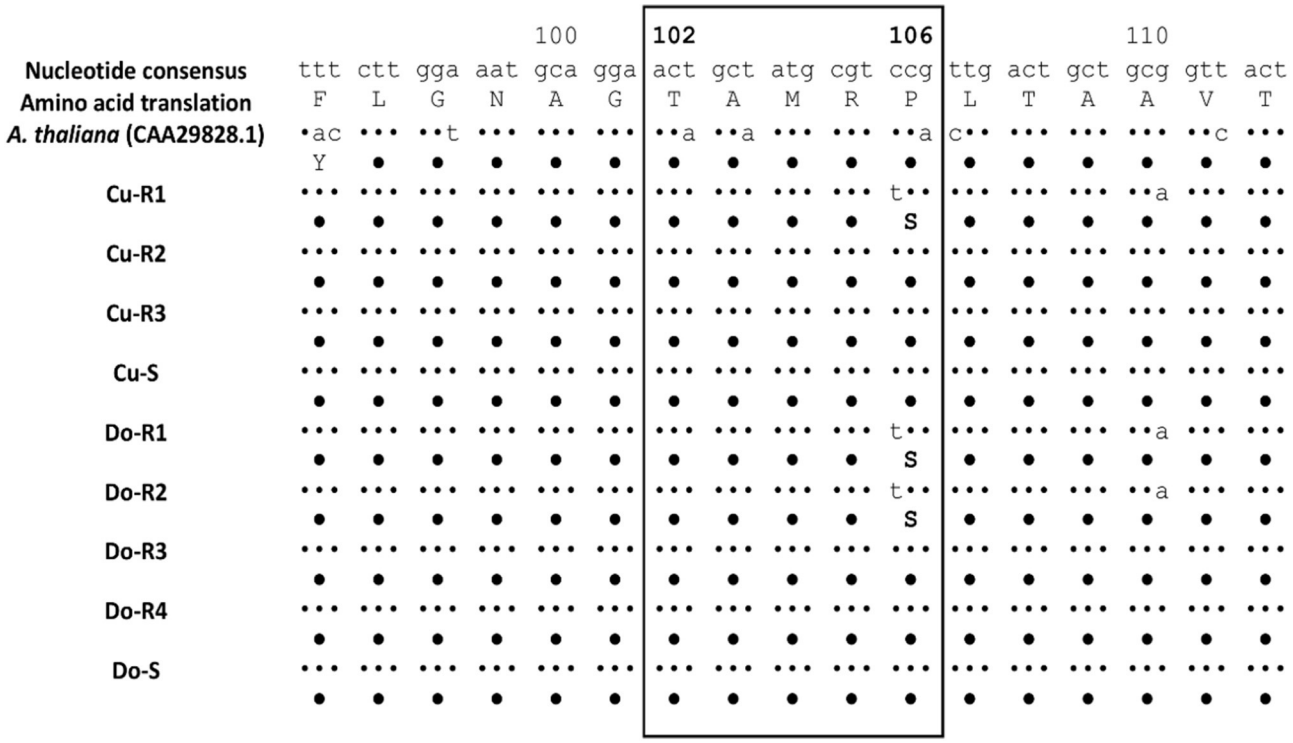

FIGURE 6 | Partial protein sequence alignment of the EPSPS gene of resistant and susceptible *P. hysterophorus* plants. The box comprising the region of Thr-102 and Pro-106 point mutations associated to confer glyphosate resistance. The points indicate homology between the different sequences.

TABLE 6 | Summary of glyphosate resistance mechanisms accumulated by *P. hysterophorus* accessions studied in this work.

Accessions	GR_{50}[a]	LD_{50}[a]	Absorption and translocation	Glyphosate metabolism	Enhanced EPSPS basal activity[b]	EPSPS (I_{50}[b])	Pro106Ser
Cu-R1	1370	6364	Low	High	Yes	High	Yes
Cu-R2	437	2794	Low	High	No	Low	No
Cu-R3	151	1415	Low	Medium	No	Low	No
Cu-S	56	232	High	Low	No	Low	No
Do-R1	939	4456	Low	High	Yes	High	Yes
Do-R2	547	2550	Low	High	Yes	High	Yes
Do-R3	339	1821	Low	High	No	Low	No
Do-R4	255	1242	Low	Medium	No	Low	No
Do-S	47	196	High	Low	No	Low	No

[a]*glyphosate g ae ha*$^{-1}$; [b]*glyphosate* μM.

observed in the I_{50} values, which could be explained as a lack of effective mutations in the binding site of the enzyme (Salas et al., 2015). In our case, some accessions are candidates to possessing an effective mutation (**Figure 6**, **Table 6**) or a possible EPSPS overexpression, explaining their high resistance to glyphosate compared to other accessions. We are aware of that fact, and effective research is currently in progress to characterize the EPSPS overexpression resistance mechanism involving these accessions.

Results reported here are in agreement with previous works, in which the Proline to Serine substitution was found to confer glyphosate resistance in other weed species such as *A. tuberculatus*, *C. sumatrensis*, *Echinochloa colona*; *L. perenne* spp. *multiflorum* and *L. rigidum* (Bostamam et al., 2012; González-Torralva et al., 2012, 2014; Nandula et al., 2013; Fernández et al., 2015; Han et al., 2016). However, mutations in the Pro-106 position generally provide only a low level (2–4-fold) of glyphosate resistance (Kaundun et al., 2011). Here, *P. hysterophorus* accessions that presented Pro-106 mutation had a resistance factor of >12. These three accessions (Cu-R1, Do-R1, and Do-R2) were more highly resistant to glyphosate as a result of showing different concurrent resistance mechanisms, including reduced absorption and translocation, glyphosate metabolism, and EPSPS gene mutation.

In some species, at least more than one glyphosate resistance mechanism have been reported, such as *A. tuberculatus* (Nandula et al., 2013), *L. rigidum* (Bostamam et al., 2012), *L. perenne* spp. *multiflorum* (González-Torralva et al., 2012), and *L. perenne* (Ghanizadeh et al., 2015) populations which exhibited a mutation in Pro-106 position, and a reduced translocation. Besides, other species such as *Digitaria insularis* presented a pool of mechanisms (absorption, translocation, metabolism, and EPSPS gene mutation; de Carvalho et al., 2012). The involvement of several resistance mechanisms is evident when looking at the resistance levels of accessions Cu-R2, Cu-R3, Cu-R4, Do-R3, Do-R4, and Do-R5 of *P. hysterophorus*, which did not show any mutation in the Pro-106 position. This is the first time that a mutation in the target-site has been reported in glyphosate-resistant *P. hysterophorus*.

In summary, we have confirmed resistance to glyphosate in different *P. hysterophorus* accessions harvested in the Caribbean Islands. Their resistance levels depend on the different resistance mechanisms (NTSR and TSR) that are accumulated by these accessions (**Table 6**), due to increasing selection pressure and out-crossing. The evolution of multiple mechanisms found in this resistance species is worrying. The farmers should implement manage practices such as the use of cover crops, which prevent soil erosion and allow the use of grazing, as well as the use of other non-selective herbicides in an integrated weed management (IWM) to facilitate the reduction and suppression of herbicide-resistant accessions.

AUTHOR CONTRIBUTIONS

EB, PF, and RD performed the glyphosate plant dose-response and shikimic acid accumulation. EB, PF, FB, and RD carried out the EPSPS activity assays. EB, PF, and RD did the ^{14}C-glyphosate absorption/translocation, and metabolism study. FB performed the EPSP synthase gene sequencing.

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Identifying *Chloris* Species from Cuban Citrus Orchards and Determining Their Glyphosate-Resistance Status

Enzo R. Bracamonte [1], Pablo T. Fernández-Moreno [2], Fernando Bastida [3], María D. Osuna [4],Ricardo Alcántara-de la Cruz [5], Hugo E. Cruz-Hipolito [6] and Rafael De Prado [2]*

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The *Chloris* genus is a C4 photosynthetic species mainly distributed in tropical and subtropical regions. Populations of three *Chloris* species occurring in citrus orchards from central Cuba, under long history glyphosate-based weed management, were studied for glyphosate-resistant status by characterizing their herbicide resistance/tolerance mechanisms. Morphological and molecular analyses allowed these species to be identified as *C. ciliata* Sw., *Chloris elata* Desv., and *Chloris barbata* Sw. Based on the glyphosate rate that causes 50% mortality of the treated plants, glyphosate resistance (R) was confirmed only in *C. elata*, The R population was 6.1-fold more resistant compared to the susceptible (S) population. In addition, R plants of *C. elata* accumulated 4.6-fold less shikimate after glyphosate application than S plants. Meanwhile, populations of *C. barbata* and *C. ciliata* with or without glyphosate application histories showed similar LD_{50} values and shikimic acid accumulation rates, demonstrating that resistance to glyphosate have not evolved in these species. Plants of R and S populations of *C. elata* differed in 14C-glyphosate absorption and translocation. The R population exhibited 27.3-fold greater 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS) activity tan the S population due to a target site mutation corresponding to a Pro-106-Ser substitution found in the EPSPS gene. These reports show the innate tolerance to glyphosate of *C. barbata* and *C. ciliata,* and confirm the resistance of *C. elata* to this herbicide, showing that both non-target site and target-site mechanisms are involved in its resistance to glyphosate. This is the first case of herbicide resistance in Cuba.

Keywords: 5-enolpyruvyl shikimate-3-phosphate synthase, glyphosate translocation, herbicide resistance mechanisms, Pro-106 mutation, tall windmill grass

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INTRODUCTION

The use of herbicides is the most common weed control method (Délye, 2013; Fernández-Moreno et al., 2017b). However, herbicide resistance has caused this method to be quickly undermined. This scenario is the result of evolutionary adaptations in a target weed to herbicide applications (Powles and Yu, 2010; Beckie and Harker, 2017). Glyphosate [(N-phosphonomethyl)-glycine]

is one of the most widely used herbicides, although it is also an herbicide with many cases of resistance (37 glyphosate-resistant species; Shaner et al., 2012; Bracamonte et al., 2016; Heap, 2017). This herbicide is systemic, non-selective and is used post-emergence, and it inhibits the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene (EC 2.5.1.19), triggering the catalysis of shikimate-3-phosphate and phosphoenolpyruvate (PEP) to form 5-enolpyruvyl-3-phosphate, an important step in the biosynthesis of aromatic amino acids in plants (Schönbrunn et al., 2001).

The mechanisms conferring glyphosate resistance are grouped into two major groups (Sammons and Gaines, 2014). Target-site resistance (TSR) can involve EPSPS gene mutations or EPSPS gene amplification. TSR was revealed to be caused by point mutations in the EPSPS gene with substitutions at Thr-102 and Pro-106 (Yu et al., 2015; Alcántara-de la Cruz et al., 2016b). Pro-106 substitutions have been found in several weeds (González-Torralva et al., 2012; Alarcón-Reverte et al., 2015; Fernandez et al., 2015), conferring low resistance levels to glyphosate on the order of 2- to 5-fold, while a double mutation (Thr-102 and Pro-106) increases resistance levels. Gene amplification is an adaptation also confers resistance to glyphosate (Gaines et al., 2013). The additional EPSPS produced from the amplified gene copies enables plants to survive higher glyphosate doses (Gaines et al., 2013; Chen et al., 2015; Yu et al., 2015). Non-target-site resistance (NTSR) mechanism results from reduced absorption and/or translocation, increased vacuolar sequestration, and metabolism to non-toxic compounds, causing less glyphosate transport to the EPSPS via the xylem and phloem (Délye, 2013). NTSR has being described as the most common mechanism of resistance to glyphosate and can confer unpredictable resistance (Powles and Yu, 2010). Similar to TSR, NTSR has been found to be a mechanism involved in resistance in many weeds (de Carvalho et al., 2012; Ge et al., 2012; Rojano-Delgado et al., 2012; Vila-Aiub et al., 2012).

The genus *Chloris* Sw. (Poaceae: Chloridoidae) is a C_4 photosynthetic species distributed in tropical and subtropical regions (Molina and Agrasar, 2004). It has also been found in semi-arid areas inhabiting semi-natural grasslands and rural habitats such as roads and barren places (Cerros-Tlatilpa et al., 2015). The genus comprises 50–60 species in both hemispheres (Molina and Agrasar, 2004; Barkworth, 2007). Species of this genus have great economic and ecological importance worldwide because they are a source of forage, resist drought, increase soil fertility, require low inversion, and can be used as plant cover to protect soil from rain-driven erosion (Michael et al., 2012). However, some of them could be considered invasive weed species (Cerros-Tlatilpa et al., 2015).

Studies of herbicide resistant weeds in Cuba are scarce because of lack of knowledge of the issue. This situation is similar to the Dominican Republic, in which studies have already begun to be carried out (Bracamonte et al., 2016). Unfortunately, growers have already started to have many weeds in their citrus groves that are not controlled at the recommended field dose of glyphosate (720 g ae ha^{-1}). Given that weed control strategies in larger commercial fields are absolutely the focus of glyphosate applications at the post-emergence growth stage, scientific confirmation is necessary. In this way, this study could define the species that are evolving toward to glyphosate resistance or even tolerant species to this herbicide.

An accurate assessment of taxonomic identity is a prerequisite to addressing population and individual plant-based functional studies. This is particularly true in the case of highly diverse genera, for which taxonomic and nomenclatural complexities generally arise, as is the case in *Chloris* (Molina and Agrasar, 2004).

This work aimed to characterize suspicious glyphosate-resistant populations of three different *Chloris* species in Cuba. Studies were conducted to (1) establish their taxonomical identity based on morphological and molecular analyses, (2) evaluate their resistance/tolerance levels, and (3) determine the mechanisms involved.

MATERIAL AND METHODS

Plant Material and Experimental Conditions

In 2014, our research group (Dr. Rafael De Prado) together with the Weed Science group of the Ministry of Agriculture of Cuba (Dr. Jorge Cueto), prospected for *Chloris* species in citrus orchards in central Cuba. These fields had been repeatedly treated with glyphosate (5 L ha^{-1}, 36% w/v) continuously for over 10 years, and sometimes received more than one application per year (Cueto, personal communication).

Mature seeds of three suspicious glyphosate-resistant populations of *Clhoris* species (treated = T) were harvested separately in in citrus orchards from Arimao and Ceiba, in Cienfuegos Province, from at least 20 plants that had been survived to the last glyphosate treatment. Seeds from a population of each species from nearby locations with no known records of exposure to glyphosate were also collected (non-treated = NT).

The seeds were germinated in containers using a substrate of sand/peat (1:2 v/v), covered with parafilm, and placed in a growth chamber at temperatures of 28/18°C (day/night), with a 16 h photoperiod (850 μmol m^{-2} s^{-1}) and 80% humidity. Subsequently, the seedlings from each population of the different *Chloris* species were transplanted individually into pots (1 plant per pot) containing the same substrate and placed in a growth chamber under the conditions described above. Furthermore, 20–30 plants from each population were placed in a greenhouse until flowering and fruiting.

Morphometric Study and Taxonomic Identity

Different taxonomically relevant morphological traits of inflorescences and caryopses were measured in greenhouse-grown plants of each species of the *Chloris* populations. The examined traits of inflorescences were the number of racemes, raceme length and spikelet density (number of spikelets per cm of raceme). For the spikelets, we examined the length and width

of the lower and upper glumes, the number of sterile florets, the length of hairs surrounding the callus, the length and width of the lemma of the fertile floret (fertile lemma), the presence and length of hairs on or adjacent to the keel and on the margins of fertile lemma, the length of the palea of the fertile floret, the lemma length and width of the basal sterile floret, the lemma length of any additional sterile floret, and the presence and length of awns on lemmas. The characteristics of the caryopses were length, width, thickness, shape, and length of the embryo mark. The shape of caryopses was quantified as the variance in their three dimensions, each relative to length (Thompson et al., 1993). This dimensionless shape index varies between 0 for a perfect sphere and 0.22 for a disk- or needle-shaped item. Based on the above morphological characters, the three pairs of study populations were identified to the species level according to Molina and Agrasar (2004), and the nomenclature followed IPNI (2017).

Molecular Characterization of the *Chloris* Species by AFLP Primer Analysis

Twenty-four accessions from the *Chloris* spp. were employed as the study material. Genomic DNA was extracted from fresh young leaves of eight individual plants per species (four T and four NT), using the Speedtools Plant DNA Extraction kit (Biotools). The DNA concentration was measured using a NanoDrop ND 1000 spectophotometer. DNA was diluted to a final concentration of 10 ng/μL.

Twelve AFLP primer pairs were used [E36-M48 (E-ACC M-CAC); E36-M60 (E-ACC M-CTC); E37-M49 (E-ACG M-CAG); E38-M50 (E-ACT M-CAT); E40-M61 (E-AGC M-CTG); E35-M49 (E-ACA M-CAG); E36-M49 (E-ACC M-CAG); E35-M61 (E-ACA M-CTG); E40-M62 (E-AGC M-CTT); E32-M60 (E-AAC M-CTC); E33-M50 (E-AAG M-CAT)]. The reaction mix contained 10 ng template DNA, 2.5 U Taq DNA polymerase, 40 pmol primer, 200 μM dNTPs, 2.5 mM $MgCl_2$, and 10 mM Tris-HCl all in a volume of 20 μl. The optimized thermal cycling conditions were 2 min at 94°C, followed by 40 cycles of 94°C for 25 s, 56°C for 25 s, 72°C for 25 s and a final extension at 72°C for 7 min. AFLP fragments were resolved in 25-cm gels (0.25 mm spacer thickness). Electrophoresis and detection were performed on a two-dye, model 4300 LICOR automated DNA Sequencer. Digital AFLP gel images were scored to obtain binary (band presence/absence) data using the SAGA GENERATION 2 software program.

Data clustering was conducted for AFLPs with the NTSYS-pc-2.2 software (Rohlf, 2000) using Jaccard's coefficients to define unweighted pair-group (UPGMA) dendograms. A principal coordinate analysis (PCA) was also performed with the NTSYS-pc program.

Dose-Response Assays

Plants of each *Chloris* population were sprayed at the 3–4 leaf growth stage. Glyphosate applications were applied with a laboratory chamber (SBS-060 De Vries Manufactering, Hollandale, MN) equipped with 8002 flat fan nozzle delivering 200 L ha^{-1} at 250 KPa at the height of 50 cm. The following glyphosate (Roundup®, 360 g ae L^{-1} as isopropylamine salt) rates were used: 0, 62.5, 125, 250, 500, 1,000, 2,000, 3,000, and 4,000 g ae ha^{-1}. The experiment was design using nine replications per rate and was repeated twice. Plants were cut down at the soil surface 21 days after application (DAT).

Shikimic Accumulation Assay

Fifty mg of fresh tissue (4 mm leaf disks) were harvested from the youngest fully expanded leaf at the 3–4 leaf growth stage from 15 plants per population. Shikimic acid accumulation was determined according to Hanson et al. (2009). The glyphosate concentrations used were: 0, 500, and 1,000 μM. Sample absorbance was measured in a Beckman DU-640 spectrophotometer at 380 nm. The test was performed in triplicate on five treated and non-treated plants of each biotype in a completely random design. Results were expressed in mg of shikimic acid g^{-1} fresh tissue.

Absorption and Translocation

This study was carried out in the two *C. elata* populations. ^{14}C-glyphosate (American Radiolabeled Chemicals, Inc., USA) was added to the commercial herbicide to prepare a solution with a specific activity of 0.834 kBq μL^{-1}. The final glyphosate concentration corresponded to 360 g ae ha^{-1} in 200 L ha^{-1}. Plants were harvested at 24, 48, 48, 72, and 96 h after ^{14}C-glyphosate treatment (0.834 kBq/plant). Five plants per populations at each time evaluated in a completely random design were handled according to Fernández-Moreno et al. (2017b). Radioactivity was analyzed by liquid scintillation spectrometry (LSS) in a Beckman LS 6500 scintillation counter (Beckman Coulter Inc. Fullerton, USA) during 10 min per sample. Percentage of ^{14}C-glyphosate absorbed was expressed as [kBq in combusted tissue/(kBq in combusted tissue + kBq in leaf washes)] × 100.

Translocation of ^{14}C-glyphosate in plants of the two *C. elata* populations was visualized using a phosphor imager (Cyclone, Perkin-Elmer, Waltham, MA, USA).

Glyphosate Metabolism

Six plants by each *C. elata* population at 3–4 leaf growth stage, were treated with 300 g ae ha^{-1} of glyphosate (as described in the dose-response assays) in a completely randomized design. Untreated plants were used as controls. Leaf tissues were washed with distilled water at 96 HAT, flash-frozen in liquid nitrogen, and stored at −40°C until use. Following the methodology described by Rojano-Delgado et al. (2010), glyphosate and its metabolites [aminomethyl phosphonate (AMPA), glyoxylate, sarcosine, and formaldehyde] were determined by reversed polarity capillary electrophoresis using a 3D Capillary Electrophoresis Agilent G1600A instrument equipped with a diode array detector (DAD, wavelength range 190–600 nm). Standard compounds used (glyphosate, AMPA, sarcosine, formaldehyde, and glyoxylate), were provided by Sigma-Aldrich, Spain. Glyoxylate naturally produced (untreated plants) was subtracted from the average of glyoxylate produced from glyphosate metabolism (treated plants) for each population.

EPSPS Enzyme Activity Assays

Leaf tissue of the *C. elata* populations (three samples of 5 g each) were ground to fine powder in liquid nitrogen a chilled mortar. The enzyme activity was extracted according to the protocol described by Sammons et al. (2007). The basal EPSPS activity in the extract was measured using a Modified Lowry Kit for Protein Determination (Sigma-Aldrich, Madrid, Spain) in accordance with the manufacturer's instructions. The specific EPSPS activity was determined using the EnzCheck Phosphate Assay Kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions, to determine the inorganic phosphate release. The glyphosate concentrations used were: 0, 0.1, 1, 10, 100, and 1,000 μM. The EPSPS activity was measured during 10 min at 360 nm in a spectrophotometer (Beckman DU-640) to determine the amount of phosphate (μmol) released μg of total soluble protein $(TSP)^{-1}$ min^{-1} and expressed as a percentage with respect to the control (without glyphosate). The experiment was repeated three times for each samples.

EPSPS Gene Sequence

Young tissue (100–200 mg) was collected from 10 plants of each *C. elata* population and stored at −80°C for RNA extraction. Total RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA was then treated with TURBO DNase (RNase-Free; Ambion, Warrington, UK) to eliminate any DNA contamination. cDNA synthesis was carried out using 2 μg of total RNA and M-MLV (Moloney Murine Leukemia Virus) Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) in combination with oligo $(dT)_{12-18}$ and random nonamers (Amersham Biosciences, Amersham, UK) according to the manufacturer's instructions. To amplify the EPSPS gene, primers previously designed by Perez-Jones et al. (2007) (forward: 5′ A GCTGTAGTCGTTGGCTGTG 3′; reverse: 5′ GCCAAGAAAT AGCTCGCACT 3′), and de Carvalho et al. (2012) (forward: 5′ TAGTACAGCCAAAAGGGCAGTC-3′; reverse: 5′ GCCGT TGCTGGAGGAAATTC 3′) were used. These primers expand a 120-bp fragment of the EPSPS gene that contains the mutation site described as conferring resistance to glyphosate. The PCR reactions were carried out using cDNA from 50 ng of total RNA, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 μM of each primer, 1× buffer, and 0.625 units of a 100:1 enzyme mixture of non-proofreading (*Thermus thermophilus*) and proofreading (*Pyrococcus furiosus*) enzymes (BIOTOOLS, Madrid, Spain) in a final volume of 25 μL. All PCR reactions were completed in duplicate, and the cycling conditions were as follows: 94°C for 3 min, 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, with a final extension cycle of 72°C for 10 min. An aliquot of the PCR product was loaded onto a 1% agarose gel to confirm the correct band amplification. The remainder of the PCR product was then purified using ExoSAP-IT® for PCR Product Clean-Up (USB, Ohio, USA) as indicated by the manufacturers. Five purified PCR products per population were sequenced (STAB VIDA, Caparica, Portugal).

Statistical Analysis

Dose-response and EPSPS enzyme activity data were subjected to non-linear regression analysis to determine the amount of glyphosate needed to reduce the fresh weight (GR_{50}), increase the mortality (LD_{50}), and inhibit the EPSPS activity (I_{50}) by 50% in each *Chloris* population using the three-parameter log-logistic function: $y = ([(d) / 1+(x/g)^b])$ where y is, depending on the analysis, the above ground fresh weight, survival, or EPSPS-activity expressed as the percentage of the non-treated control, d is the parameter corresponding to the upper asymptote, b is the slope, g is the GR_{50}, LD_{50}, or I_{50}, and x (independent variable) is the glyphosate rate. Regression analyses were conducted using the *drc* package in the R program version 3.2.5 (Ritz et al., 2015). Resistance indexes (RI = R/S) were computed as R-to-S GR_{50}, LD_{50}, or I_{50} ratios.

An analysis of variance (ANOVA) was conducted to test for differences between populations in the different assays. When needed, differences between means were separated using the Tukey HSD test at $P < 0.05$. Model assumptions of the normal distribution of errors and homogeneous variance were graphically inspected. The ANOVAs were conducted using the Statistix (version 9.0) (Analytical Software, USA) software.

RESULTS

Morphometric Study and Taxonomic Identity

Based on the examined morphological traits, the populations studied were identified as *C. ciliata* Sw., *C. elata* Desv., and *Chloris barbata* Sw. Only the last species listed is an annual species, and it can be easily separated from the other two species by its long-awned lemmas, the glabrous keel of the fertile lemma and the presence of hairs flanking the keel. In addition, caryopses of *C. barbata* were clearly more elongated in shape than those of the remaining species, as indicated by the higher values of the seed shape index. Distinctive traits of *C. ciliata* include a low number of racemes in the inflorescences, more than two sterile florets per spikelet, long keel hairs and relatively short awns. Compared to the other two species, racemes of *C. elata* plants were consistently longer and their fertile lemmas were shorter in length, showing much longer marginal hairs. In addition, the embryo mark in caryopses was shorter in this species (**Table 1**).

Molecular Characterization of the Genus *Chloris*

A cluster analysis using UPGMA methods classified the *Chloris* populations into two major groups (I and II), thus providing complementary information to the morphological analysis. Group I contained all samples of *C. ciliata* whereas Group II consisted of two subgroups, II-1 including *C. elata*, and II-2 including *C. barbata*. It is noting that the cluster analysis did not separate T and NT populations of *C. ciliata* or *C. barbata*, while the T and NT populations of *C. elata* were clearly separated (**Figure 1**).

TABLE 1 | Comparison of morphological traits from inflorescences and caryopses for the three pairs of studied *Chloris* populations, and their taxonomic identity at the species level.

Species		*C. ciliata* T	*C. ciliata* NT	*C. elata* T	*C. elata* NT	*C. barbata* T	*C. barbata* NT
Number of racemes composing inflorescences		5 (5–7)	5 (4–5)	17–28	11–27	13 (11-15)	12–18
Racemes length		54.6 ± 6.8	76.0 ± 12.0	117.0 ± 20.9	96.0 ± 16.9	71.2 ± 8.5	73.0 ± 9.2
Number of spikelet per cm raceme		14 (14–14)	14 (12–14)	11 (9–11)	14 (12–15)	12 (12–13)	13 (12–13)
Number of sterile florets in spikelets		3 (3–4)	4 (3–4)	2 (2–2)	2 (2–2)	2 (2–2)	2 (2–2)
Length of hairs surrounding spikelet callus		0.66 ± 0.21	0.79 ± 0.12	0.18 ± 0.03	0.40 ± 0.06	0.95 ± 0.08	0.97 ± 0.12
Fertile floret, lemma	Length	2.68 ± 0.19	2.65 ± 0.18	1.98 ± 0.11	1.87 ± 0.10	2.63 ± 0.12	2.54 ± 0.10
	Max width	2.14 ± 0.19	2.18 ± 0.20	1.36 ± 0.09	1.56 ± 0.10	1.18 ± 0.17	1.13 ± 0.14
	Hairy (H)/glabrous (G) keel	H	H	H	H	G	G
	Length of keel hairs	1.20 ± 0.15	1.30 ± 0.15	0.67 ± 0.05	0.70 ± 0.06	–	–
	Length of hairs flanking keel	–	–	–	–	0.57 ± 0.08	0.58 ± 0.14
	Length of marginal hairs	1.13 ± 0.15	1.42 ± 0.26	2.22 ± 0.16	2.09 ± 0.10	1.40 ± 0.13	1.40 ± 0.06
	Awn length	1.63 ± 0.26	1.71 ± 0.35	3.14 ± 0.36	2.53 ± 0.27	6.97 ± 0.60	5.53 ± 1.08
Fertile floret, palea	Length	2.44 ± 0.14	2.55 ± 0.20	1.82 ± 0.09	1.58 ± 0.09	2.45 ± 0.14	2.35 ± 0.23
Basal (first) sterile floret, lemma	Length	1.66 ± 0.14	1.73 ± 0.18	1.09 ± 0.08	1.05 ± 0.05	1.38 ± 0.12	1.37 ± 0.05
	Max width	2.15 ± 0.21	2.23 ± 0.23	0.92 ± 0.10	0.97 ± 0.08	1.27 ± 0.28	1.33 ± 0.08
	Awn length	1.58 ± 0.26	1.72 ± 0.23	2.83 ± 0.15	2.22 ± 0.29	6.37 ± 1.23	5.33 ± 1.89
Second sterile floret, lemma	Length	1.14 ± 0.17	1.08 ± 0.15	0.50 ± 0.05	0.53 ± 0.04	1.29 ± 0.09	1.33 ± 0.08
	Awn length	A	A	A	A	5.22 ± 0.81	3.82 ± 0.88
Glumes	Upper, length	2.57 ± 0.17	2.78 ± 0.07	3.10 ± 0.06	3.25 ± 0.20	2.82 ± 0.08	2.78 ± 0.12
	Upper, width	1.00 ± 0.19	0.92 ± 0.13	0.53 ± 0.05	0.45 ± 0.14	0.60 ± 0.06	0.55 ± 0.05
	Lower, length	1.75 ± 0.15	1.73 ± 0.08	2.07 ± 0.13	2.03 ± 0.14	1.78 ± 0.08	1.80 ± 0.06
	Lower, width	0.83 ± 0.10	0.78 ± 0.04	0.73 ± 0.08	0.80 ± 0.06	0.58 ± 0.08	0.45 ± 0.05
Caryopsis	Length	1.43 ± 0.08	1.35 ± 0.12	1.12 ± 0.04	1.03 ± 0.05	1.45 ± 0.05	1.40 ± 0.00
	Width	0.70 ± 0.04	0.58 ± 0.09	0.57 ± 0.04	0.60 ± 0.00	0.45 ± 0.03	0.43 ± 0.03
	Thickness	0.58 ± 0.04	0.53 ± 0.07	0.45 ± 0.03	0.47 ± 0.03	0.33 ± 0.03	0.31 ± 0.02
	Length of the embryo mark	0.83 ± 0.08	0.77 ± 0.09	0.59 ± 0.07	0.62 ± 0.13	0.93 ± 0.03	0.90 ± 0.05
	Seed shape index[a]	0.069 ± 0.008	0.078 ± 0.001	0.068 ± 0.005	0.055 ± 0.008	0.121 ± 0.004	0.122 ± 0.005

[a] *Variance of the three dimensions, each relative to length.*

Means and standard deviations are given for quantitative traits, and modal (if any), minimum and maximum values for qualitative traits. Sample size n = 6. Linear dimensions are given in mm. A, absent.

Dose-Response Assays

The fresh weight reduction by 50% (GR_{50}) for the NT and T populations of *C. elata* was achieved at 88.3 and 542.1 g ae ha^{-1}, respectively, i.e., T populations were 6.1-fold more resistance to glyphosate than the NT population. The LD_{50} values of the T populations exhibited 15-fold resistance than the NT population of *C. elata*. In contrast, GR_{50} and LD_{50} values in both *C. barbata* and *C. ciliata* species were not different between T and NT populations (**Table 2,Figure 2**).

Shikimic Acid Accumulation

No differences were found between the exposure of leaf disks to 500 or 1,000 μM of glyphosate. At 1000 μM, the NT population of *C. elata* presented 4.6-fold more shikimic than the T population. However, the T populations of *C. barbata* and *C. ciliata* showed no differences, accumulating only 1.13 and 1.07-fold more shikimic acid, respectively, than their respective NT populations (**Figure 3**).

It was determined that *C. barbata* and *C. ciliata* exhibited natural tolerance to glyphosate, and the T and NT populations of *C. elata* were renamed resistant (R) and susceptible (S) to glyphosate, respectively. In the following assays, we focused in *C. elata.*

Absorption and Translocation

Total ^{14}C-glyphosate recovery was 93.2 and 94.3% for the R and S populations of *C. elata*, respectively (data not shown). ^{14}C-glyphosate absorption increased slowly in the first 72 HAT. At this time, population S had absorbed 30% of glyphosate, while population R had only absorbed 22%. The maximum glyphosate absorption rate was observed between 72 and 96 HAT, which was the two-fold higher in the S population (64%) than in the R population (33%; **Figure 4A**).

In both populations, ^{14}C-glyphosate levels in treated leaves of *C. eleta* declined from 24 to 96 HAT, with the rate of movement out of the treated leaf greater and faster in the S population than in R population. The initial amount quantified of 73% at 24 HAT in the treated leaf decreased to 38% at 96 HAT in S population. Conversely, the ^{14}C-glyphosate was retained mainly in the leaf treated in the R population, dropping from 81 to 62% at 24 and

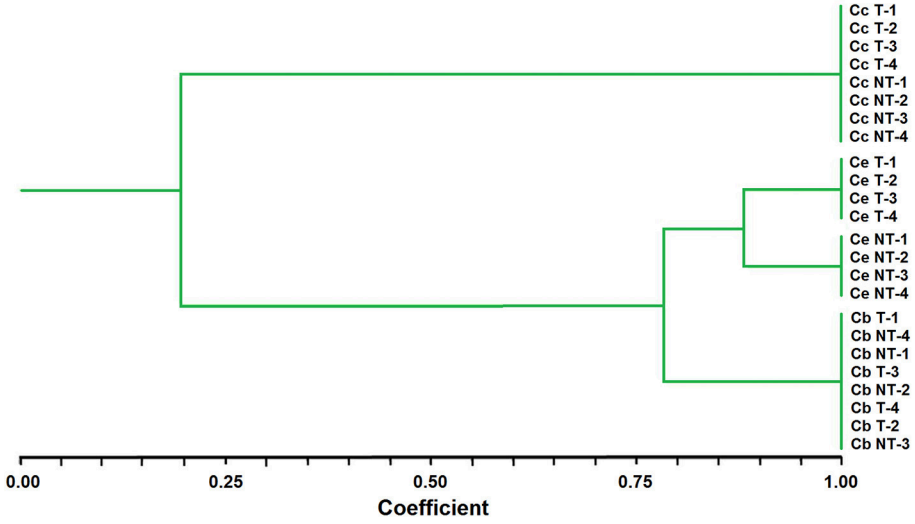

FIGURE 1 | Genetic similarities among *Chloris* species after UPGMA analysis performed with AFLP marker data. Ce, *Chloris elata*; Cb, C. *barbata*; and Cc, *C. Ciliata;* T, plants with glyphosate history applications; NT, non-treated plants.

TABLE 2 | Glyphosate rates required for 50 % reduction fresh weight (GR_{50}) and survival (LD_{50}) expressed as percentage of the mean untreated control of *Chloris* species.

Species	Status[a]	GR_{50} (g ae ha^{-1})	RI[b]	*P*	LD_{50} (g ae ha^{-1})	RI[b]	*P*
C. elata	T	542.1 ± 31.3	6.1	0.001	2277.7 ± 245.1	15.0	0.001
	NT	88.3 ± 4.8			151.6 ± 24.8		
C. barbata	T	217.2 ± 19.6	1.1	0.241	889.4 ± 71.5	1.1	0.382
	NT	198.9 ± 21.9			820.2 ± 79.0		
C. ciliata	T	263.1 ± 19.2	1.1	0.159	912.7 ± 68.6	1.1	0.297
	NT	231.3 ± 37.4			875.2 ± 59.3		

±Standard error (n = 10). [a]Status: T, populations with glyphosate history applications; and NT, non-treated populations with glyphosate. [b]RI, Resistance index (R/S) calculated using the corresponding ED_{50}, or LD_{50} values of the resistant populations respect to the susceptible one.

96 HAT, respectively. An average of 32 and 30% of the glyphosate translocated reached the remaining shoot tissue and roots at 96 HAT in the S population, whereas in R population it was only of 20 and 18%, respectively (**Figures 4B,C**).

The ^{14}C-glyphosate visualization by phosphor imaging revealed differences in the distribution between the S and R populations of *C. elata.* There was a difference in the translocation of glyphosate from treated leaves to shoots and roots, and the S population translocated higher amounts of ^{14}C-glyphosate compared to the R population (**Figure 5**).

Glyphosate Metabolism

For glyphosate absorbed into the R and S *C. elata* populations, much of the herbicide was unaltered in plants by 96 HAT. At this time, 89.4 and 91.0% of the applied herbicide remained as glyphosate in plants of the R and S populations, respectively. The levels of AMPA were 7.2 and 6.4%, while glyoxylate levels reached 3.4 and 2.6% in the R and S plants, respectively. For both AMPA and glyoxylate, these differences between the R and S populations were non-significant ($P = 0.8741$ for AMPA, and $P = 0.6318$ for glyoxylate).

EPSPS Enzyme Activity Assays

The R population was 27.3-fold more resistant than the S population. The basal enzyme activity showed no differences between populations with 0.0987 to 0.0937 mmol mg^{-1} TPS^{-1} min^{-1} for the R and S populations, respectively (**Figure 6**).

Sequencing of the EPSPS Gene

A total of 120 bp of the EPSPS gene of the R and S *C. elata* populations was sequenced. The fragments were aligned and numbered based on a published EPSPS sequence of *Leptochloa virgata* (GenBank: KX425854) (Alcántara-de la Cruz et al., 2016c). Protein alignment of the predicted EPSPS fragments from R and S populations of *C. elata* showed 91.6 and 92.5% protein similarity, respectively, to that of *L. virgata*. The R population of *C. elata* showed an amino acid substitution at position 106 consisting of a proline to serine change. The substitution resulted in a TCG (serine) codon instead of a CCG (proline) codon (**Figure 7**).

DISCUSSION

The three *Chloris* populations from citrus orchards in Cuba were identified as *C. barbata, C. ciliata,* and *C. elata*. The number of species of *Chloris* recognized by different authors in Mexico and in the nearby Caribbean Islands is variable (Barkworth, 2007; Cerros-Tlatilpa et al., 2015). In these regions, the most frequent species are *C. ciliata, C. elata, C. barbata,* and *C. virgata* (Cerros-Tlatilpa et al., 2015). In Cuba, 12 species of the *Chloris* genus have been found (Catasús-Guerra, 2002).

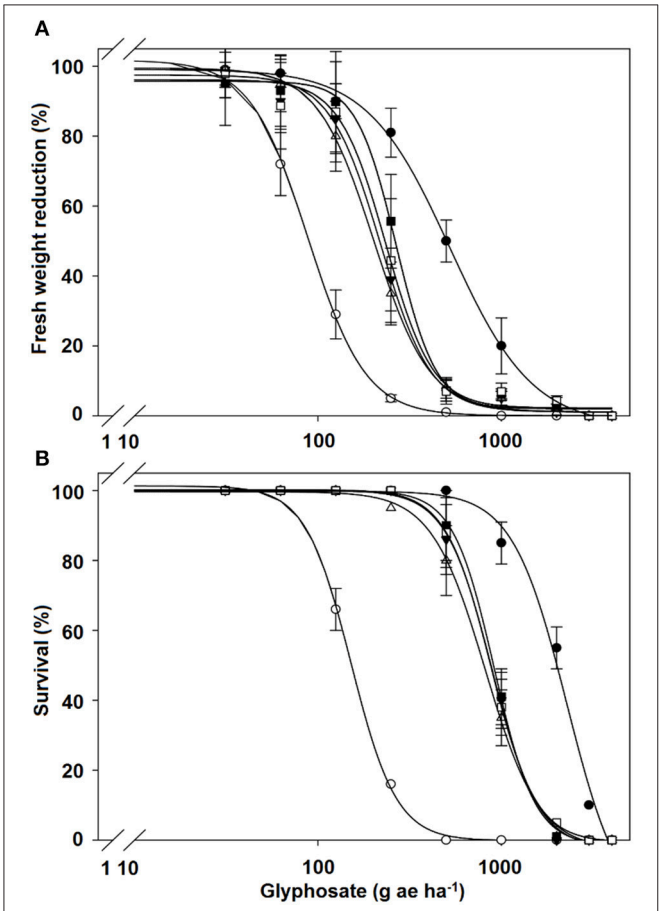

FIGURE 2 | Glyphosate **(A)** dose-response on fresh weight reduction and **(B)** % survival expressed as percentage respect to untreated control of the treated (T) (•) and non-treated (NT) (○) populations of *Chloris elata*; T (▼) and NT (△) populations of *C. barbata*; and T (■) and NT (□) populations of *C. ciliata*. Vertical bars are ± standard errors ($n = 9$).

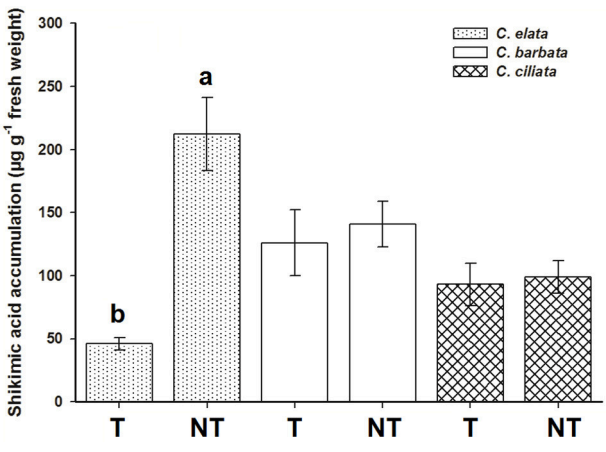

FIGURE 3 | Shikimic acid accumulation in *Chloris* spp. plants at 1,000 µM glyphosate concentration. T = plants with glyphosate history applications; and NT = non-treated plants. Vertical bars are ± standard errors ($n = 5$). Different letters shown differences at 95% by the Tukey's test.

The AFLP-based classification of *Chloris* populations revealed molecular-based relationships between three basic entities, which closely matched the morphology-based identification of three different species. The selected AFLP markers can be useful candidates in the pursuit of disentangling phylogenetic relationships among *Chloris* species. However, although these markers allowed to separate the T and NT populations of *C. elata,* they cannot be used to detect glyphosate resistance, because could produce biased results due to fragment-size homoplasy (Caballero et al., 2008).

To characterize the glyphosate susceptibility in *Chloris* species, it is important to consider the innate tolerance to this herbicide that has been observed in some species of the genus. Depending on the species studied, the LD_{50} (% survival plant) values can vary between 515 and 703 g ae ha^{-1} (Ngo et al., 2017a,b). These values are lower than those we have found for *C. barbata* and *C. ciliata*, including the populations never exposed to this herbicide, demonstrating an innate tolerance to glyphosate in these two species. Similar results were described for *Chloris polydactyla* from Brazil, where even some accessions with no history of applications presented lower susceptibility to glyphosate than those accessions with a history of glyphosate applications (Barroso et al., 2014). Innate tolerance has been well studied in grass weeds (Fernández-Moreno et al., 2016), and leguminous species (Cruz-Hipolito et al., 2009, 2011; Rojano-Delgado et al., 2012; Alcántara-de la Cruz et al., 2016a; Mao et al., 2016). The mechanism proposed is a lack of ^{14}C-glyphosate absorption and/or translocation in tolerant plants compared to susceptible ones.

C. elata has a different profile than *C. barbata* and *C. ciliata*, and its GR_{50} and LD_{50} values demonstrate a clear quantitative difference between those plants harvested from T fields compared to those plants from NT fields. The lower shikimic accumulation by the T *C. elata* population (4.9 times) compared to the NT population showed the greatest resistance level of this species, similar to other *Chloris* species, such as *C. elata* (5.4) from Brazil (Brunharo et al., 2016) and *C. virgata* (2.0–9.7) and *Chloris truncata* (2.4–8.7) from Australia (Ngo et al., 2017a,b). When glyphosate is applied via foliar application, the EPSPS enzyme is inhibited, and there is a rapid accumulation of shikimate (Shaner et al., 2005). The amount of glyphosate in the NT population of *C. elata* determined the inhibition of EPSPS and rapid shikimate accumulation demonstrating the high susceptibility (S) of this population. Therefore, the T population of *C. elata* was characterized as resistant (R) to glyphosate, and the populations T and NT of *C. barbata* and *C. ciliata* are tolerant to this herbicide. These results are reflected in those obtained in dose response assays. For this reason, we continued to study the glyphosate resistance mechanisms only in the case of *C. elata*.

To date, few cases of reduced glyphosate absorption and/or translocation have been studied as a mechanism of resistance in the genus *Chloris,* and the results are contradictory. ^{14}C-glyphosate studies on *C. virgata* and *C. truncata* do not

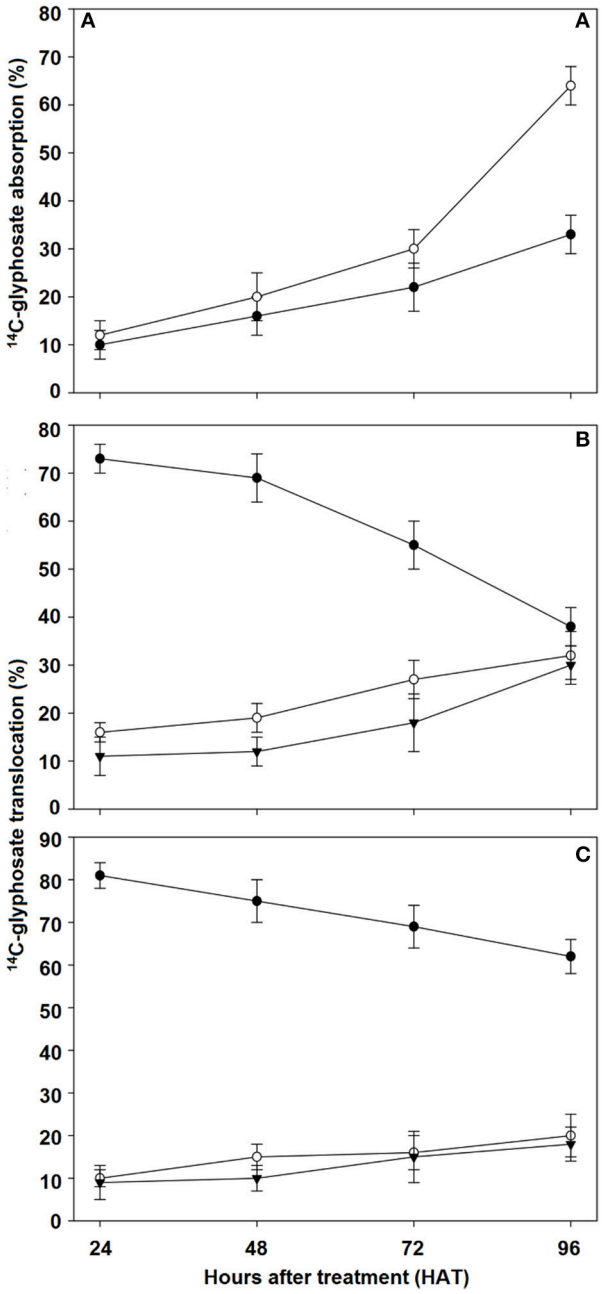

FIGURE 4 | ^{14}C-glyphosate absorption and translocation in susceptible (S) and resistant (R) *Chloris elata* populations from 24 to 96 h after glyphosate treatment. **(A)** ^{14}C-glyphosate absorption in S (○) and R (•) plants. ^{14}C-glyphosate translocation in S **(B)** and R **(C)** plants. Treated leaf (•), remaining shoot tissue (○), and roots (▼). Vertical bars are ± standard errors ($n = 5$).

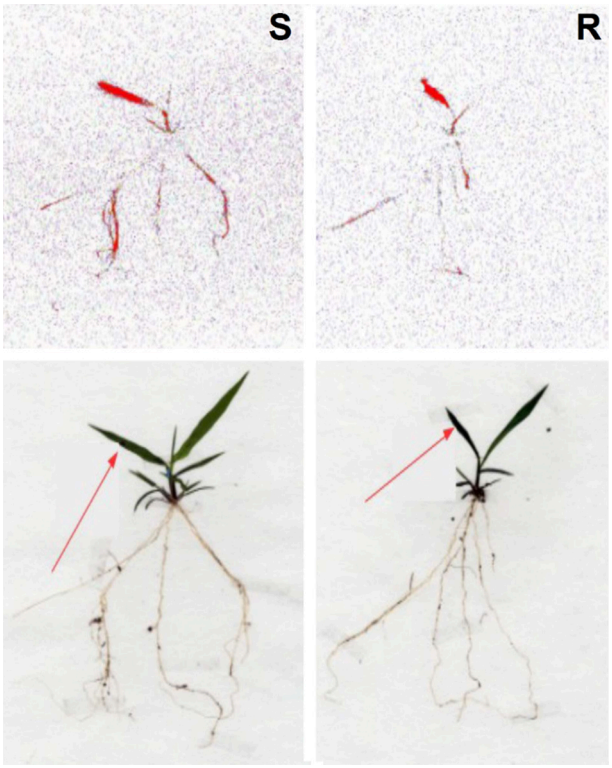

FIGURE 5 | Translocation of ^{14}C-glyphosate in susceptible **(S)** and resistant **(R)** *Chloris elata* plants at 96 h after application. The highest concentration of ^{14}C-glyphosate is highlighted in red. Arrows indicate the treated leaf.

show significant differences in the absorption and subsequent translocation of the herbicide, and the resistance was determined by mechanisms within the target site (Ngo et al., 2017a,b). However, another study on *C. elata* shows that lower glyphosate absorption and translocation in the R population are the only mechanisms involved in its glyphosate resistance (Brunharo et al., 2016). In our case, the R *C. elata* population collected in Cuba shows a resistance mechanism similar to that previously found for the species. Thus, ^{14}C-absorption and translocation are higher in the S population than in the R population. These results suggest that less absorption and translocation contributed to the resistance to glyphosate in R *C. elata* plants.

Glyphosate metabolism has thus far not been identified as a major mechanism of resistance in plants, but is likely the result of plants not succumbing to glyphosate because of the expression of another resistance mechanism (Sammons and Gaines, 2014; Fernández-Moreno et al., 2017a). Only in a few cases has metabolism been demonstrated to be a secondary mechanism in glyphosate resistance, because in these cases, other major mechanisms are involved (Bracamonte et al., 2016). Our research substantiates that of other studies, with <90% of the absorbed glyphosate remaining unaltered in R and S plants of *C. elata*. It is likely that the ability of grass weeds to metabolize glyphosate is diminished once EPSPS is inhibited (González-Torralva et al., 2012; Fernandez et al., 2015). Considering the small extent of glyphosate metabolism, the significance of this result is unlikely biologically meaningful in the resistance to glyphosate in *C. elata*.

The I_{50} values were significantly different between the *C. elata* populations. The R population exhibited a high resistance level compared to the S population. The results with high I_{50} values and low shikimic acid values, as has already been explained and demonstrated in other studies, are associated with alterations

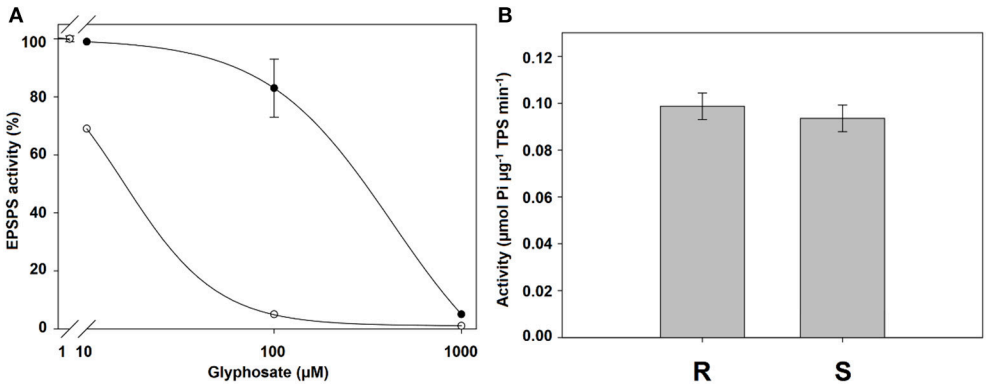

FIGURE 6 | (A) EPSPS enzyme activity expressed as percentage of the untreated control in leaf extracts of plants from glyphosate-susceptible (○) and -resistant (•) *Chloris elata* plants. **(B)** Basal EPSPS activity for *C. elata* populations. Vertical bars are ± standard errors ($n = 3$).

Position	99	100	101	**102**	103	104	105	**106**	107	108	109
Consensus sequence	AAT	GCA	GGA	ACC	GCT	ATG	CGC	**CCG**	TTG	ACC	GCT
L. virgata (KX425854)	AAC	GCT	GGA	ACT	GCG	ATG	CGC	**CCA**	TTG	ACG	GCT
Amino acid translation	Asn	Ala	Gly	Thr	Ala	Met	Arg	**Pro**	Leu	Thr	Ala
C. elata S	Asn	Ala	Gly	Thr	Ala	Met	Arg	**Pro**	Leu	Thr	Ala
C. elata R	Asn	Ala	Gly	Thr	Ala	Met	Arg	**Ser**	Leu	Thr	Ala

FIGURE 7 | Partial alignment of nucleotides and amino acid sequences of EPSPS genes between glyphosate-susceptible and -resistant *Chloris elata* populations and *Leptochloa virgata* (GenBank: KX425854). The yellow color indicates nucleotide changes among species. The green color indicates an amino acid substitution at 106 position from Proline (CCG) to Serine (TCG). Box includes the 102 (blue) and 106 (orange) positions (amino acid number based on the start codon (ATG) of *Arabidopsis thaliana*, corresponding to point mutations associated for conferring glyphosate resistance.

in the gene encoding the herbicide target protein (Sammons and Gaines, 2014; Yu et al., 2015). Then, the TSR mechanism could be involved in the resistance to this species. Similar results have been shown in other weed species, including *L. virgata* (Alcántara-de la Cruz et al., 2016c), *Lolium multiflorum* (Salas et al., 2015), and *L. rigidum* (Fernandez et al., 2015). In these cases, higher I_{50} values, as well as the higher basal activity of EPSPS, were found in the resistant populations compared to the susceptible populations. It was thought that an overexpression of EPSPS played a role as a resistance mechanism (Ngo et al., 2017a). However, there were no significant differences in the basal activity of EPSPS between R and S populations of *C. elata*, precluding the involvement of such a mechanism.

The EPSPS sequence alignment showed only a mutation point at position Pro-106-Ser in the R *C. elata* population. Four substitutions in this genomic EPSPS position (Pro-106-Ser, Pro-106-Thr, Pro-106-Ala, and Pro-106-Leu), have been reported in mono- and dicotyledonous weeds, endowing resistance to glyphosate (Sammons and Gaines, 2014). A mutation to a different amino acid at this point causes a structural change in the target site, shifting the other amino acids toward the inhibitor by reducing the available space (Healy-Fried et al., 2007). These explain the resistance of the R population of *C. elata* at a molecular level. Some grassweed species which have shown a mutation at Pro-106 position are: *C. virgata* (Ngo et al., 2017a), *Echonoclhoa colona* (Alarcón-Reverte et al., 2015), *L. virgata* (Alcántara-de la Cruz et al., 2016c), *L. rigidum* (Fernandez et al., 2015), and *Poa annua* (Cross et al., 2015), among others.

CONCLUSIONS

Morphological- and molecular-based analysis allowed the identification of the three *Chloris* species collected in citrus orchards from central Cuba. *C. barbata* and *C. ciliata* were characterized as being innately tolerant to glyphosate, and *C. elata* was identified as resistant to this herbicide. The last species had non-target site (reduced absorption and translocation) and target site (Pro-106-Ser mutation) resistance mechanisms to glyphosate.

These results confirm the first case of herbicide resistance in Cuba and strongly suggest that species of the *Chloris* genus can be either resistant or tolerant to glyphosate, supporting the previous reports of both glyphosate statuses in this genus.

AUTHOR CONTRIBUTIONS

EB and RDP: Idea and designed the experiments; EB, PF-M, MO, FB, HC-H, and RA-dlC: Performed the research. PF-M, FB, MO, RA-dlC, and RDP; Analyzed the results. All authors contributed to write and approve the manuscript.

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From tolerance to resistance: Mechanisms governing the differential response to glyphosate in *Chloris barbata*

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Species of the genus *Chloris* have a certain level of natural tolerance to glyphosate. Variable susceptibility to glyphosate and the mechanism(s) governing tolerance/resistance to this herbicide were characterized into two putative glyphosate-resistant *Chloris barbata* populations (R1 and R2), collected in Persian lime orchards from Colima State, Mexico, where received glyphosate (720 g ae ha^{-1}) applications 7-year (3-4 times per year). The resistant (R) populations were compared to one non-treated (referred as S) population. The amounts of glyphosate to reduce fresh weight and cause mortality by 50% of R populations were 4.2-6.4 times higher than the S population. The latter one accumulated from 4.3-5.2 times more shikimic acid than the R populations. There were no differences in ^{14}C-glyphosate uptake between R and S *C. barbata* populations, but the R plants translocated at least 12% less herbicide to the rest of plant and roots 96 hours after treatment. Insignificant amounts of glyphosate were metabolized to AMPA and glyoxylate in both R and S plants. The 5- enolpyruvylshikimate-3-phosphate synthase gene of the R *C. barbata* populations contained the Pro-106-Ser mutation, giving them a resistance up to 12-14.7 times greater at the target site in comparison with the S plants. The continuous applications of glyphosate increased tolerance and induced resistance in *C. barbata.* The Pro-106-Ser mutation governs the resistance to glyphosate of the R1 and R2 *C. barbata* populations, but the impaired translocation could be contributing to the resistance. These results confirm the first case of glyphosate resistance evolved in this species.

Keywords: 5-enolpyruvylshikimate-3-phosphate synthase; herbicide resistance; impaired translocation; Pro-106-Ser mutation; purpletop chloris.

Research Article

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From tolerance to resistance: mechanisms governing the differential response to glyphosate in *Chloris barbata*

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Abstract

BACKGROUND: Susceptibility and the mechanism (s) governing tolerance/resistance to glyphosate were characterized in two putative-glyphosate-resistant *Chloris barbata* populations (R1 and R2), collected in Persian lime orchards from Colima State, Mexico, comparing them with one non-treated population (referred to as S).

RESULTS: Glyphosate doses required to reduce fresh weight or cause mortality by 50% were 4.2–6.4 times higher in resistant populations than in the S population. The S population accumulated 4.3 and 5.2 times more shikimate than the R2 and R1 populations, respectively. There were no differences in ^{14}C-glyphosate uptake between R and S populations, but the R plants translocated at least 12% less herbicide to the rest of plant and roots 96 h after treatment. Insignificant amounts of glyphosate were metabolized to aminomethyl phosphonate and glyoxylate in both R and S plants. The 5-enolpyruvylshikimate-3-phosphate synthase gene of the R populations contained the Pro106-Ser mutation, giving them a resistance 12 (R2) and 14.7 (R1) times greater at target-site level compared with the S population.

CONCLUSION: The Pro106-Ser mutation governs the resistance to glyphosate of the R1 and R2 *C barbata* populations, but the impaired translocation could contribute to the resistance. These results confirm the first case of glyphosate resistance evolved in this species.

Keywords: 5-enolpyruvylshikimate-3-phosphate synthase; herbicide resistance; impaired translocation; Pro106-Ser mutation; purpletop chloris

1 INTRODUCTION

Mexico is the second largest producer and main exporter of Persian lime (*Citrus latifolia* Tan.).[1] Veracruz (Gulf of Mexico) is the main producer of this citrus fruit, but to cover the large volume of exports, its production has been extended to states with similar environmental characteristics, such as Colima, Guerrero, Michoacán and Oaxaca (Pacific Coast).[2] Tropical rainfall conditions allow continuous Persian lime production all year round, but also favor the emergence of weeds, such as *Bidens pilosa*,[3] *Eleusine indica*[4] and *Leptoclhoa virgate*,[5] which have been identified as being resistant to glyphosate. They do not have a great impact on yield, but weeds can make cultivation difficult.[6] Therefore, the presence of weeds can have a significant impact on production costs if they remain uncontrolled.[7] Weed management in citrus orchards in Mexico includes mechanical, chemical (mainly glyphosate-based herbicides)[8] and combined methods.[7]

Although glyphosate [(*N*-phosphonomethyl)glycine] belongs to the herbicide group with the greatest increase in resistance cases in recent years, it is the most widely used non-selective and systemic herbicide.[9,10] This herbicide deactivates the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (EC 2.5.1.19) enzyme in the shikimic pathway by interrupting the catalysis of shikimate-3-phosphate and phosphoenolpyruvate (PEP) to form 5-enolpyruvylshikimate-3-phosphate, an important step in the biosynthesis of aromatic amino acids in plants.[10,11] Because glyphosate is the only herbicide that inhibits EPSPS,

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shikimate accumulation is considered an unequivocal indication of the effect of glyphosate on susceptible plants.[12]

Species of the genus *Chloris* are distributed worldwide. They have slow initial growth, mainly in shady conditions or at low temperatures, but can be found in semiarid areas.[13] In Mexico, *Chloris* species are invasive weeds found in both altered and conserved areas.[14] *Chloris barbata* (L.) Sw. is widely distributed in the coastal states of Mexico,[14] coinciding with the main citrus-producing regions. It is worth noting that species of the genus *Chloris* have a certain level of natural tolerance to glyphosate in comparison with species of other genera,[15–18] which allows these species to survive and reproduce after field herbicide applications lethal to wild plants of other species.[19] The survival of weed individuals after repeated application of the same herbicide or action mechanism is due to evolutionary adaptations resulting in herbicide resistance.[20]

Total or partial glyphosate resistance/tolerance may be due to alterations in the target-site represented by single or double mutations in the conserved region of the *EPSPS* gene,[3,21] *EPSPS* gene duplication[22] or both.[4,23] Reduced uptake and/or impaired translocation and degradation of glyphosate into non-toxic substances as well as hypersensitive reactions have also been reported as non-target site resistance mechanisms contributing to the resistance to this herbicide.[24–26]

Chloris barbata individuals survived the widely used standard dose of 720 g acid equivalent (ae) ha^{-1} of glyphosate in citrus-production systems from Colima. Loss of susceptibility to glyphosate in *C. barbata* may be due to an increase in its innate tolerance or the evolution of resistance mechanisms. The aims of this study were to characterize the different levels of glyphosate susceptibility in two putative resistant *C. barbata* populations, collected in Persian lime orchards from Colima, and the mechanism (s) governing their resistance/tolerance to this herbicide.

2 MATERIAL AND METHODS

2.1 Biological material and experimental conditions

Seeds from resistant *C. barbata* populations (R1 and R2), collected from at least 20 plants that survived the final glyphosate treatment of 720 g ae ha^{-1} in 2014, were harvested in two Persian lime orchards of the Valenzuela farm (18°54′52′′N, 103°51′37′′W), in the municipality of Tecoman, Colima. Fields from which seed samples were taken were at least 600 m apart, and had a 7-year history of glyphosate application (three or four times per year). Glyphosate was always applied when weeds were well grown or setting seeds in these orchards. Weed management was also complemented with hand mowing three or four times per year 2–3 weeks prior to herbicide treatment. Seeds of a non-treated population (referred as S) were collected near the Persian lime groves (18°59′38′′N, 103°50′46′′W).

Seeds were sown in trays (15 × 15 × 8 cm) with peat substrate moistened to field conditions and covered with parafilm. The trays were taken to a growth chamber calibrated for 26/18 °C day/night, 16 h photoperiod at 850 $\mu mol^{-2}\ s^{-1}$ of light intensity, and 60% relative humidity (RH). The seedlings were transplanted into 3 L pots (five plants per pot) containing a sand/peat mixture (1, 1 v/v), before placing them back in the growth chamber. They were watered daily until glyphosate treatment.

A screening test was conducted on the R populations to eliminate susceptible individuals from the field-collected seed. Fifteen pots were treated with 720 g ae ha^{-1} of glyphosate (Roundup Energy 45% w/v, Monsanto, Madrid, Spain) using a Generation III Research Track Sprayer (DeVries Manufacturing Inc., Hollandale, MN, USA) chamber equipped with an 8002EVS nozzle (TeeJet, Spraying System Spain, S.L., Madrid, Spain) calibrated to deliver 200 L ha^{-1} at 200 kPa at a height of 50 cm. Surviving individuals were grown to maturity, bulked and allowed to produce seeds.

The new purified seeds were germinated under the conditions described above, but seedlings were transplanted individually into 250 mL pots. *Chloris barbata* plants with three or four true leaves were used for all experiments.

2.2 Dose–response

Plants from the three (S, R1 and R2) *C. barbata* populations were sprayed with the following increasing doses of glyphosate: 0, 62.5, 125, 250, 500, 1000, 2000, 3000 and 4000 g ae ha^{-1} in a treatment chamber. Three weeks after application, survival was assessed and the fresh shoots of the aerial part of plants were harvested and weighed. The experimental design included 10 replicates per dose and was repeated twice. Data were expressed in percentages.

2.3 Shikimic accumulation assay

Samples of 50 mg of young leaf tissue (4 mm leaf discs) were taken. Shikimic acid accumulation was determined according to Shaner *et al.*[27] The glyphosate concentrations used were: 0, 100, 500 and 1000 µM. Sample absorbance was measured using spectrophotometer (Beckman DU-640, Beckman Instruments Inc., Fullerton, CA, USA) at 380 nm. The experiments had a completely random design using three tissue samples from each *C. barbata* population per glyphosate concentration and they were repeated twice. Results were expressed in mg of shikimic acid per g of fresh tissue.

2.4 Uptake and translocation

Chloris barbata plants of the S, R1 and R2 populations were treated with a ^{14}C-glyphosate [glycine-2-^{14}C] (specific activity 273.8 MBq $mmol^{-1}$, American Radiolabeled Chemicals, Inc., St. Louis, MO, USA) + commercial glyphosate solution. The final glyphosate concentration corresponded to 360 g ae ha^{-1} in 200 L ha^{-1} which contained a specific activity of 50 000 dpm μL^{-1} (equivalent to 0.834 kBq μL^{-1}). Twenty plants per population were treated with one drop (1 µL $plant^{-1}$) of solution on the adaxial surface of the first or second leaf. The plants were handled according to Alcántara-de la Cruz *et al.*[5] at 24, 48, 72 and 96 h after treatment (HAT) (five plants per population at each time evaluated in a completely random design). Radioactivity of ^{14}C was analyzed by liquid scintillation spectrometry in a scintillation counter (Beckman LS-6500, Beckman Coulter Inc.) for 10 min per sample. Radioactive values in dpm were used to calculate the percentages of ^{14}C-glyphosate recovered, taken up, and translocated.

2.5 Metabolism

Five plants from each *C. barbata* population were treated with 360 g ae ha^{-1} of glyphosate. Untreated plants were used as controls. Leaf tissues were washed with distilled water at 4 days after treatment, flash-frozen in liquid nitrogen, and stored at −40 °C until use. The methodology described by Rojano-Delgado *et al.*[28] was used to determinate the percentage of glyphosate and its metabolites (aminomethyl phosphonate (AMPA), glyoxylate, sarcosine and formaldehyde) by reversed polarity capillary electrophoresis using a 3D Capillary Electrophoresis Agilent G1600A instrument equipped with a diode array detector (DAD; wavelength range 190–600 nm). Standard compounds used were

Table 1. Glyphosate dose/concentration required to kill individuals of a population (LD_{50}), reduce the fresh weight (GR_{50}) and/or inhibit the EPSPS activity (I_{50}) by 50% in glyphosate-resistant (R1 and R2) and -susceptible (S) *Chloris barbata* populations

Population	GR_{50} (g ae ha^{-1})	RI	LD_{50} (g ae ha^{-1})	RI	I_{50} (μM)	RI
R1	613.9 ± 92.8	4.8	2295.6 ± 391.9	4.2	112.9 ± 19.5	12.0
R2	810.4 ± 136.1	6.4	3127.5 ± 476.6	5.7	138.3 ± 26.7	14.7
S	127.1 ± 4.1	–	544.9 ± 22.4	–	9.4 ± 0.9	–

RI, Resistance indexes (R/S) calculated using LD_{50}, GR_{50} or I_{50} of the respective resistant population and LD_{50}, GR_{50} or I_{50} of the susceptible population. Values are given ±95% CI.
[a] $n = 10$ per glyphosate dose.
[b] $n = 3$ technical replicates per glyphosate concentration.

provided by Sigma-Aldrich (Madrid, Spain). Glyoxylate naturally produced (untreated plants) was subtracted from the average of glyoxylate produced from glyphosate metabolism (treated plants) for each biotype. The experiment had a completely randomized design and was repeated twice. Data were expressed as percentages from the total of glyphosate plus metabolites that were recovered.

2.6 EPSPS enzyme activity

One 5 g sample of young tissue was collected from 20–30 plants from each *C. barbata* population. Samples were ground to a fine powder in liquid nitrogen in a chilled mortar, and enzyme extraction was performed following the protocol described by Sammons *et al.*[29] The total soluble protein (TSP) in the extract (basal activity in absence of glyphosate) was measured using a Kit for Protein Determination (Sigma-Aldrich) following the manufacturer's instructions. Specific EPSPS activity was assayed in the presence of glyphosate (0, 0.1, 1, 10 100 and 1000 μM) using the EnzChek Phosphate Assay Kit (Invitrogen, Carlsbad, CA, USA). EPSPS activity was measured for 10 min at 360 nm in a spectrophotometer (Beckman DU-640) to determine the amount of inorganic phosphate (μmol) released, as measured in μg^{-1} TSP min^{-1}, and expressed as a percentage relative to the control (absence of glyphosate). The experiment was repeated twice with three technical replications at each glyphosate concentration.

2.7 *EPSPS* gene sequencing

Total RNA was extracted from 10 plants from each *C. barbata* population following the methodology described by Pistón.[30] RNA integrity was verified in 0.8% agarose gel and quantified in a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). First-strand complementary DNA (cDNA) synthesis was carried out with 1 μg of RNA per sample using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The degenerate primers BpF13 (5′-TTGCCYGGRTCMAAGTCTTT-3′) and BpR11 (5′-GTCCCAASTATCACTRTGTTC-3′), designed from *EPSPS* gene sequences of different weed species,[3] were used to amplify a 639 bp fragment. The polymerase chain reaction (PCR) conditions described by Alcántara-de la Cruz[3] were followed in a total volume of 25 μL per reaction [50 ng of cDNA, 0.2 μM of each primer, 0.2 mM dNTP mix (PE Applied Biosystems; Life Technologies S.A., Spain), 2 mM $MgCl_2$, 1× buffer and 0.625 units of a 100: 1 enzyme mixture of non-proofreading (*Thermus thermophilus*) and proofreading (*Pyrococcus furiosus*) polymerases (BIOTOOLS, Madrid, Spain)]. Each PCR was carried out in triplicate. Ten microliters of PCR product was checked by 1% agarose gel, and 15 μL were reserved for cloning. Amplicons were cloned into competent cells of *Escherichia coli* (DH5α) and positive transformants were confirmed through PCR using the universal primers M13F (5′-CGCCAGGGTTTTCCCAGTCACGAC-3′) and M13R (5′-TCACACAGGAAACAGCTATGAC-3′).[3] The plasmids were purified using the ilustra plasmidPrep Mini Spin kit (GE Healthcare, Little Chalfont, UK), and Sanger sequencing of two or three clones per plant was performed by STAB VIDA (Caparica, Portugal). Sequence assembly was carried out using the SeqMan Pro 11.0 (DNASTAR, USA) and Geneious 8.1.8 (Biomatters Ltd., New Zealand) software programs.

2.8 Statistical analysis

Data percentages for fresh weight reduction, survival and EPSPS enzyme activity were submitted to a non-linear regression analysis to find out the amount of glyphosate needed to reduce the fresh weight (GR_{50}), cause mortality (LD_{50}), and inhibit EPSPS activity (I_{50}) by 50% of each *C. barbata* population. The log-logistic equation used is: $Y = c + \{(d - c)/[1 + (x/g)^b]\}$,[31] where Y is the percentage of fresh weight, mortality and/or EPSPS enzyme inhibited relative to the control; c and d are the lower and upper limits, respectively, of the curve; b is the slope at the inflection point (i.e., GR_{50}, LD_{50} or I_{50}); and x is the glyphosate dose. Regression analyses were conducted using the *drc* package with program R version 3.2.5.

Data of shikimic acid, basal EPSPS activity, uptake, translocation and metabolism were subjected to analysis of variance (ANOVA). Model assumptions of normal distribution of errors and homogeneous variance were graphically inspected. Differences with $P < 0.05$ were considered significant and Tukey's test was conducted for means comparison.

3 RESULTS

3.1 Dose–response

Different glyphosate susceptibility levels were corroborated between S and R *C. barbata* populations. The LD_{50} for the S population was 544.9 g ae ha^{-1}, whereas the values for the R1 and R2 populations were 2295.6 and 3127 g ae ha^{-1}, respectively. At 1000 g ae ha^{-1}, < 20% of S plants survived, and at 2000 g ae ha^{-1}, all plants of this population died. The fresh weight reduction (GR_{50}) of S population was 4.8 and 6.4 times larger in relation to the R1 and R2 populations, respectively (Table 1, Fig. 1).

3.2 Shikimic acid accumulation

The amount of shikimic acid in the absence of glyphosate was similar between the R and S populations. Once treated, the three *C. barbata* populations accumulated shikimic acid as the glyphosate doses gradually increased. The accumulation was

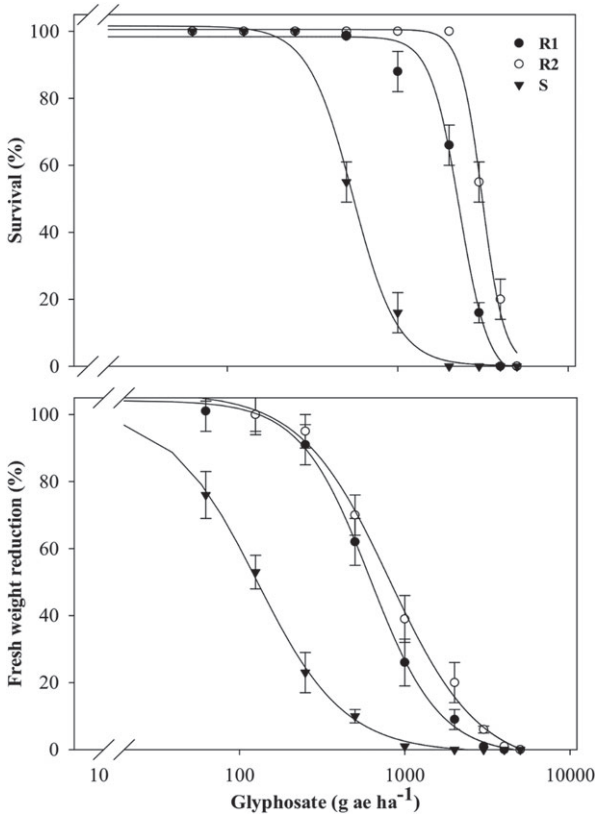

Figure 1. Dose–response curves of survival (upper) and fresh weight reduction (lower) in glyphosate-susceptible and -resistant *Chloris barbata*. Vertical bars represent the standard error of the mean ($n = 10$ per glyphosate dose).

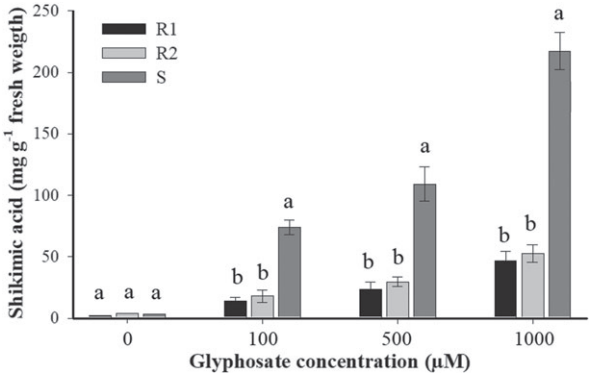

Figure 2. Shikimic acid accumulation of glyphosate-susceptible and -resistant *Chloris barbata* plants at different glyphosate concentrations. Groups of bars with the same letter above them are not different using the Tukey test at 95%. Vertical bars represent the standard error of the mean ($n = 3$ per glyphosate concentration).

markedly higher in the S plants than the R plants. The latter (R1 and R2) presented similar patterns of shikimic acid accumulation. At 1000 µM glyphosate, S plants accumulated between 4.3 and 5.2 times more shikimic acid (~ 225 mg shikimic acid g^{-1} fresh weight) than R1 and R2 plants, respectively (Fig. 2).

3.3 Uptake and translocation

The uptake of ^{14}C-glyphosate ranged from 15.3 to 20.9% at 24 HAT, and from 30.4 to 32.9% at 96 HAT. There were no significant

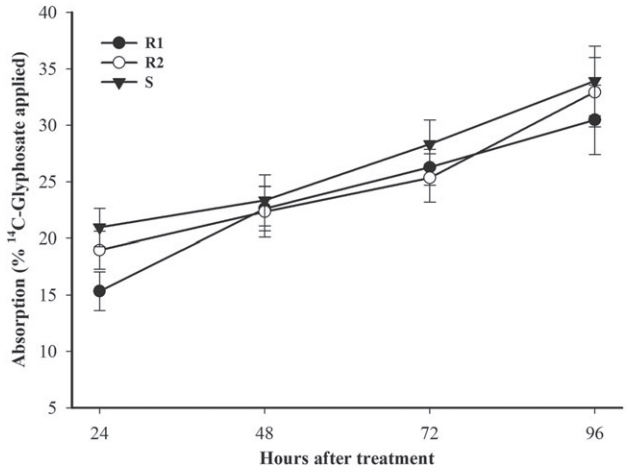

Figure 3. ^{14}C-Glyphosate uptake in glyphosate-susceptible and -resistant *Chloris barbata* plants 24–96 h after treatment. Vertical bars represent the standard error of the mean ($n = 5$ per time evaluated).

differences in ^{14}C-glyphosate uptake between R and S *C. barbata* populations (Fig. 3); however, there were differences in the ^{14}C-glyphosate translocation. S plants moved at least 12% more herbicide to the rest of plant and root system at 96 HAT, compared with R plants. Therefore, we found ~ 4–7% more radio-labeled herbicide in the rest of plant, and up to 5–9% in the roots of S plants (Table 2).

3.4 Glyphosate metabolism

No differences were found between R and S *C. barbata* populations with regards to glyphosate metabolites. At 96 HAT, ~ 95% of glyphosate was not metabolized by the plants. The levels of AMPA and glyoxylate ranged from 2.8% to 4.3% and 0.8% to 1.3%, respectively; sarcosine was not detected (Table 2).

3.5 EPSPS enzyme activity

The amount of glyphosate needed to inhibit the EPSPS activity by 50% (I_{50}) in the S population was 9.4 µM. Regarding this value, the R1 and R2 populations were 11.0 and 14.7 times more resistant, respectively, than the S population (Table 1, Fig. 4). Meanwhile the specific activity of EPSPS in absence of herbicide showed no differences between the R and S *C. barbata* populations, with averages of 0.033 ± 0.005 (R1), 0.036 ± 0.080 (R2) and 0.032 ± 0.008 (S) µmol Pi µg^{-1} TSP min^{-1}.

3.6 Sequencing of the *EPSPS* gene

The sequences of the *EPSPS* genes from *C. barbata* populations were aligned and numbered based on the known *EPSPS* sequence of *Leptochloa virgata*. Different single nucleotide polymorphisms (SNPs) between species were observed, but with a homology >97% at the protein level. The R populations of *C. elata* showed an amino acid substitution (R1 = 8 and R2 = 7) at the 106 position consisting of a proline (CCG) to serine (TCG) (Fig. 5). Of the 10 individuals sequenced per R population, eight and seven plants of the R1 and R2, respectively, presented the mutation Pro106-Ser, i.e., 80% and 70% of the sample size analyzed.

4 DISCUSSION

The resistance to glyphosate of the R1 and R2 *C. barbata* populations was confirmed, because their GR_{50} values were close to

Table 2. Percentages of translocation and metabolism of ^{14}C-glyphosate in resistant (R1 and R2) and susceptible (S) *Chloris barbata* populations at 96 h after treatment

	Translocation[a] (from % absorbed)			
Population	Treated leaf	Rest of plant		Root system
R1	67.9 ± 4.1 a	18.8 ± 1.8 b		13.3 ± 2.6 b
R2	64.2 ± 3.9 a	21.7 ± 2.3 ab		15.1 ± 2.4 b
S	53.4 ± 3.7 b	25.4 ± 3.3 a		21.2 ± 2.1 a
	Metabolites (%)			
	Glyphosate	AMPA	Glyoxilate	Sarcosine
R1	94.6 ± 1.4	4.3 ± 0.7 a	1.1 ± 0.3	ND
R2	96.4 ± 1.7	2.8 ± 0.5 b	0.8 ± 0.3	ND
S	95.3 ± 1.5	3.4 ± 0.5 ab	1.3 ± 0.5	ND

[a] Percent of herbicide-labeled absorbed. Entries within a column followed by the same letter are not statistically different using the Tukey test at 95%. Values are given ± the standard error of the mean ($n = 5$). AMPA, aminomethylphosphonic acid.

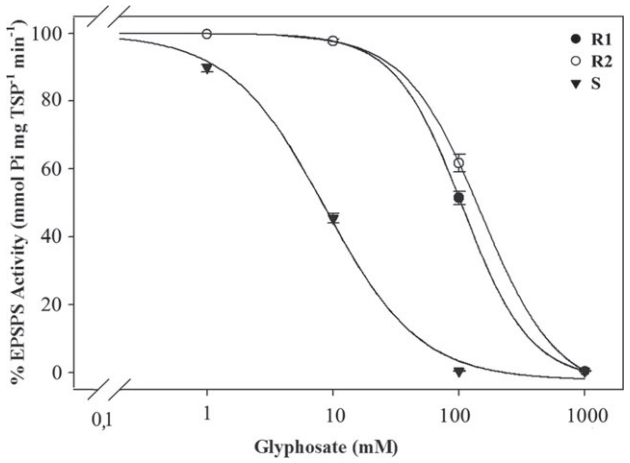

Figure 4. EPSPS enzyme activity expressed as percentage of the untreated control in leaf extracts of glyphosate-susceptible and -resistant *Chloris barbata* plants. Vertical bars represent the standard error of the mean ($n = 3$ per glyphosate concentration).

the standard dose of 720 g ae ha^{-1}, and the LD_{50} values were above this value (Table 1). In addition, the S *C. barbata* population showed LD_{50} value (545 g ae ha^{-1}) close to the standard dose, evidencing a certain innate tolerance level in this species. Sensitive populations of *C. elata*, *C. ciliata*, *C. truncata* and *C. virgata* also recorded high degrees of tolerance to glyphosate with LD_{50} ranging from 515 to 1385 g ae ha^{-1} of glyphosate.[16–18] Taking into account that controlling a weed population requires at least twice its estimated LD_{50},[3] the standard dose of 720 g ae ha^{-1} used is not enough to control the S *C. barbata* population (Fig. 1). Depending on the cropping system, weed species, and infestation level, the effective glyphosate doses may range from 700 to 2100 g ae ha^{-1}.[32] It would be more appropriate to apply doses >1100 g ae ha^{-1} (twice the LD_{50} of population S) to achieve an acceptable control of *C. barbata*, but the Persian lime farmers from Mexico have widely adopted the standard dose of 720 g ae ha^{-1}, because this dose controls a large number of weeds and they often have to make several glyphosate applications per year. However, this glyphosate dose results in low levels of control for *C. barbata*. Unfortunately, low herbicide doses can select for resistant phenotypes quicker than the recommended dose.[33] As a consequence, the R1 and R2 *C. barbata* populations increased their tolerance level and possibly developed resistance mechanisms against glyphosate. Therefore, alternative herbicides, such as diuron + paraquat, glufosinate, and glufosinate + indaziflam, that showed satisfactory control in glyphosate-resistant *Leptochloa virgata* populations in Persian lime orchards from Veracruz, Mexico,[8] may be applied to achieve an acceptable control of *C. barbata*.

The loss of susceptibility to glyphosate in the R *C. barbata* plants was reflected in their low accumulation of shikimic acid compared with S plants (Fig. 2). The lower shikimate accumulation in R plants indicates limited interaction of glyphosate with the EPSPS protein, and may be due to either target site or non-target site resistance mechanisms.[34] However, it is difficult to deduce the mechanism (s) involved in the resistance of each resistant *C. barbata* population based solely on shikimate accumulation, because this parameter is just a resistance indicator.[4]

^{14}C-glyphosate uptake was similar between R and S *C. barbata* plants (Fig. 3), observations that are in agreement with the results reported for *C. virgata* plants from Australia, which did not present differences in their uptake patterns.[18] However, the S *C. barbata* plants translocated slightly more ^{14}C-glyphosate than the R plants (Table 2). Any reduction in translocation negatively affects glyphosate efficiency.[35] This is a mechanism that is not completely understood, but an unknown barrier in the phloem system or in the mesophyll cells has been suggested,[10] altering the subcellular glyphosate distribution. In addition, it is suspected that potential ABC transporters could be involved in the impaired ^{14}C-glyphosate translocation.[36,37] These results suggest that translocation might be contributing to glyphosate resistance in these R *C. barbata* populations. However, it is difficult to determine the degree of their contribution to resistance, because the translocation differences were lower than those observed in R *C. elata* populations from Brazil,[38] where this mechanism played an important role. It is likely that resistance to glyphosate in *C. barbata* via reduced translocation is an evolving mechanism, because sublethal doses select genes related to non-target site mechanisms,[33] which could play an important role if steps are not taken to delay/stop the evolution of resistance.[20]

The R and S *C. barbata* plants showed similar glyphosate metabolism rates (Table 2). Glyphosate metabolism has been studied in various glyphosate-resistant and -tolerant weed

Amino acid position				100		102				106				110	
L. virgata S (KX425854)	TTG	GGG	AAC	GCT	GGA	ACT	GCG	ATG	CGG	CCA	TTG	ACG	GCT	GCT	GTA
	L	G	N	A	G	T	A	M	R	P	L	T	A	A	V
Consensus of *C. barbata*	CTT	GTT	AAT	GCA	GGA	ACC	GCT	ATG	CGC	CCG	TTG	ACC	GCT	GCA	GTT
	L	G	N	A	G	T	A	M	R	P	L	T	A	A	V
R1	L	G	N	A	G	**T**	A	M	R	**S**	L	T	A	A	V
R2	L	G	N	A	G	**T**	A	M	R	**S**	L	T	A	A	V
S	L	G	N	A	G	**T**	A	M	R	**P**	L	T	A	A	V

Figure 5. Partial alignment of nucleotides and amino acid sequences of *EPSPS* genes of glyphosate-susceptible and -resistant *Chloris barbata* populations. The yellow color indicates nucleotide changes among species. The green color indicates an amino acid change at the 106 position from proline (CCG) to serine (TCG). Box includes positions 102 and 106 corresponding to mutations confirmed to confer glyphosate resistance.

species such as: *Colognia broussonetti*,[19] *C. elata*,[16] *Echinochloa colona*,[39] *Ipomea lacunosa*[40,41] and *Lolium* species,[42] among others. However, the contribution of glyphosate metabolism as a resistance mechanism is not clear, and so far occurs at significantly lower rates in glyphosate than glyphosate-resistant transgenic crops carrying the glyphosate oxidoreductase (GOX) gene.[43] In this respect, these studies demonstrated that treated weed plants, be they susceptible, tolerant or resistant, can metabolize minimal amounts of glyphosate into non-toxic substances, which could be a biological characteristic.[16] However, metabolism does not appear to be related to resistance to glyphosate of the R1 and R2 *C. barbata* populations.

Unlike the shikimate accumulation test, differences in the enzymatic activity of EPSPS directly evidence alterations in the gene encoding the target protein.[34] The similar basal activity between R and S *C. barbata* plants suggested that there was no *EPSPS* gene amplification in the R populations, although this mechanism was characterized as the major one that conferred resistance to glyphosate in R *C. truncata* plants from Australia.[18] In the absence of differences in the *EPSPS* basal activity, the higher I_{50} values in R *C. barbata* plants (Fig. 4) reveal mutations in the *EPSPS* encoding gene.

The *EPSPS* gene sequences of the R1 and R2 *C. barbata* populations showed the mutation Pro106-Ser (Fig. 5). Several mutations in the *EPSPS* gene have been suggested as contributing to glyphosate resistance such as: Val133-Ile and Pro382-Leu in *E. indica*,[43] Asp71-Met, Ala112-Ile and Val201-Met in *Ophiopogon japonicus*, *Liriope platyphylla* and *L. spicata*,[44] and Glu91-Ala in *C. truncata*,[18] among others. However, the mutations responsible for conferring resistance to glyphosate must occur in the conserved region of the *EPSPS* gene, which includes amino acid positions 95 to 107,[4] as demonstrated in *E. coli*.[45,46] To date, only two mutations (Thr102 and Pro106) occurring in this region have been found.[3,21] Our results clearly demonstrate that the mutation Pro106-Ser was involved in the evolution of resistance to glyphosate in *C. barbata* R plants. The resistance levels associated with mutations in this position (from Pro to Ser, Ala, Leu or Thr) are well established in weeds, being two to three times higher than the recommended doses. The mutations Pro106-Ser and -Leu/-Ser were identified in glyphosate-resistant populations of *C. elata* from Cuba[16] and *C. virgata* from Australia,[17] respectively, contributing totally or partially to their resistance.

5 CONCLUSIONS

The relatively 'low doses' of glyphosate (~ 720 g ae ha^{-1}) to control weeds in Persian lime orchards from Colima increased tolerance and selected for resistance to this herbicide in *C. barbata*. The amino acid change from Pro to Ser in the 106 position of the *EPSPS* gene governs the resistance to glyphosate of the R1 and R2 populations. The impaired translocation, an evolving non-target-site resistance mechanism which is being selected by the 'low glyphosate doses', could play an important role in the resistance. Therefore, adequate doses of glyphosate and alternative herbicides must be used for the management of *C. barbata*, which contribute to delaying/stopping the evolution of glyphosate resistance. These result confirm the first case of glyphosate resistance evolved in *C. barbata*.

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